DESCRIPTION

SCAFFOLD-FREE SELF-ORGANIZED 3D SYNTHETIC TISSUE

TECHNICAL FIELD

The present invention relates to the field of regenerative medicine. More particularly, the present invention relates to a synthetic tissue capable of functioning after implantation, a method for producing the same, and use of the same. The synthetic tissue of the present invention has biological integration capability.

BACKGROUND ART

Recently, regenerative therapy has attracted attention as a novel approach to severe organ failure or intractable diseases. Regenrative therapy is a combination of genetic engineering, cell tissue engineering, regenerative medicine, and the like. Many researchers over the world are vigorously working on this important and challenging subject of research in the 21-century advanced medical practice.

The scale of the market associated with regenerative medicine (tissue engineering) is estimated as about 500 billion US dollars in the world and about 50 billion US dollars in Japan according to the material prepared by the New Energy and Industrial Technology Development Organization. Only tissue engineering products account for about 100 billionUS dolloars in the world. The regenerative medicine is greatly expected to create the next-generation industry.

The present inventors have made efforts to develop regenerative therapy in the field of musculoskeletal and cardiovascular tissues, and have reported a combination therapy of cell implantation and a growth factor administration, or a tissue implantation regeneration therapy based on tissue engineering. However, regenerative therapy based on cell or tissue implantation requires a source of autologous cells. A stable and abundant source of such cells is urgently required and important. A number of cells in musculoskeletal tissue have a high level of self-repairing ability. It has been reported that there is a stem cell among the cells of the musculoskeletal tissue.

It has been demonstrated that a cell derived from skeletal muscle (Jankowiski R.J., Huand J. et al, Gene Ther., 9:642-647, 2002), fat (Wickham M.Q. et al., Clin. Orthop., 2003, 412, 196-212), umbilical cord blood (Lee O.K. et al., Blood, 2004, 103:1669-75), tendon (Salingcarnboriboon R., Exp. Cell. Res., 287:289-300, 2002), bone marrow (Pitterger M.F. et al., Science, 284:143-147, 1999), and synovium (Arthritis Rheum. 2001 44:1928-42) is undifferentiated and has the potential to differentiate into various cells.

Conventionally, when cell therapy is performed for repair or regeneration of tissue, most research employs a biological scaffold to maintain the accumulation of cells, allow cells to grow, maintain pluripotency, protect cells from mechanical stress on a treated site, or the like. However, most scaffolds contain a biological (animal) material, a biomacromolecule material, or the like, of which influence on the safety of organism cannot be fully predicted.

A cell implanting method without a scaffold has been

reported by Kushida A., Yamato M., Konno C., Kikuchi A., Sakurai Y., Okano T., J. Biomed. Mater. Res., 45:355-362, 1999, in which a cell sheet is produced using a temperature sensitive culture dish. Such a cell sheet engineering technique is internationally appraised due to its originality. However, a single sheet obtained by this technique is fragile. In order to obtain the strength that can withstand surgical manipulation, such as implantation, a plurality of sheets need to be assembled, for example.

When a nano-biointerface technology is used to fix a temperature responsive polymer (PIPAAm) onto a plastic mold, such as a Petri dish, for cell culture, the polymer surface is reversibly changed at 31°C between hydrophilicity and hydrophobicity. Specifically, when the temperature is 31°C or more, the surface of the Petri dish is hydrophobic so that cells or the like can adhere thereto. In this situation, the cells secrete extracellular matrix (ECM; for example, adhesion molecules which are proteins having a function like a "glue") and adhere to the surface of the Petri dish, so that the cells can grow. See, Okano T., Yamada N., Sakai H., Sakurai Y., J. Biomed. Mater. Res., 1993, 27:1243-1251; Kushida A., Yamato M., Konno C., Kikuchi A., Sakurai Y., Okano T., J. Biomed. Mater. Res. 45:355-362, 1999; and Shimizu T., Yamato M., Akutsu T. et al., Circ. Res., 2002, Feb 22; 90(3):e40.

When the temperature is 31°C or less, the surface of the Petri dish is hydrophilic. The cells which have adhered to the Petri dish are readily detached, though the cells still maintain adhesion molecules. This is because the surface of the Petri dish to which the cells have adhered no longer exists at 31°C or less.

Even when such a Petri dish having a fixed temperature responsive polyer (e.g., tradename: UpCell and RepCell) is used to culture cells and detach the cells, an extracellular matrix is not appropriately provided. Thus, there has been no actually practical synthetic tissue developed. See, Okano T., Yamada N., Sakai H., Sakurai Y., J. Biomed. Mater. Res., 1993, 27:1243-1251; Kushida A., Yamato M., Konno C., Kikuchi A., Sakurai Y., Okano T., J. Biomed. Mater. Res. 45:355-362, 1999; and Shimizu T., Yamato M., Akutsu T. et al., Circ. Res., 2002, Feb 22; 90(3):e40.

WO00/51527 and WO03/024463 reported that cells are cultured on a semipermeable membrane using alginate gel. However, the resultant tissue is poorly integrated with an extracellular matrix and is not free of a scaffold. In addition, the cells in the tissue are not self organized. The tissue has no self-supporting ability. The cells no longer have a differentiation potential. The tissue loses morphological plasticity in terms of three-dimensional structure. Therefore, the tissue is not suitable for cell implantation.

Use of a scaffold is considered to be problematic in implantation therapy because of adverse side effects. Therefore, there is a demand for the advent of a scaffold-free technique.

Conventional methods for producing tissue sheets have the following drawbacks: it is not possible to produce a very large sized sheet; it is not possible to produce a sheet having biological integration in three dimensions; when a sheet is detached after sheet production, the sheet

is broken into pieces; and the like.

Therefore, there is a keen demand for a synthetic tissue, which is developed by culture processes, capable of withstanding an implantation operation, capable of being used in an actual operation.

By conventional techniques, it is difficult to isolate a synthetic tissue from a culture base material after tissue culture, and it is substantially impossible to produce a large sized tissue piece. Therefore, conventional synthetic tissues, such as tissue sheets, cannot be used in medical application in view of size, structure, mechanical strength, and the like. It is difficult to develop a synthetic tissue using conventional techniques. Therefore, unfortunately their supplies are limited.

An object of the present invention is to provide a synthetic tissue produced by cell culture, which is feasible to implantation surgery.

Specifically, an object of the present invention is to provide a synthetic tissue having a three-dimensional structure and self-supporting ability, being free of a scaffold, and maintaining a differentiation potential if the tissue possesses it.

Still another object of the present invention is to provide a method and a pharmaceutical agent for treating an injury of a tissue or the like when a replacement or resurfacing therapy is required.

DISCLOSURE OF THE INVENTION

The above-described objects were achieved in part based on the invention of the following synthetic tissue. When a cell was cultured in medium containing an extracellular matrix (ECM) synthesis promoting agent, cells and ECM produced by the cells are integrated to formed a tissue, which was readily detached from the culture dish.

The above-described objects were achieved by providing a synthetic tissue of the present invention which is free of a scaffold, has self-supporting ability, is easily formed into a three-dimensional structure, has morphological plasticity, has excellent ability to biologically adhere to surroundings, has a differentiation potential, and the like, and finding that the synthetic tissue is effective for a replacement or resurfacing therapy at an injured site.

The present invention also provides a method for producing an implantable synthetic tissue, which has biological integration and does not require assembling layers.

The above-described objects were achieved by finding that the thickness of the synthetic tissue of the present invention can be adjusted to a desired value by regulating a physical or chemical stimulus on the synthetic tissue.

The present inventors realized the formation of a three-dimensional synthetic tissue (cellular therapeutic system) comprising cultured cells (e.g., fat-derived cells, etc.) and material produced by the cells without a scaffold.

The synthetic tissue of the present invention can

be constructed into various shapes and has a sufficient strength. Therefore, it is easy to surgically manipulate (e.g., implant, etc.) the synthetic tissue of the present invention. According to the present invention, a large quantity (e.g., 10^6 to 10^8) of cells can be securely supplied to a local site by means of tissue implantation.

In the matrix, cell adhesion molecules, such as collagen (e.g., type I, type III), fibronectin, vitronectin, and the like, are present in large amounts. Particularly, the cell adhesion molecules are integrated throughout the matrix.

Therefore, the tissue has excellent ability of biologically adhesion to surroundings of the implanted site. Thus, the synthetic tissue complex biologically adheres to an implanted site tissue very quickly. In addition, by changing culture conditions, the synthetic tissue can be differentiated into a bone or cartilage tissue. The maintenance of a differentiation potential is a feature of the synthetic tissue of the present invention which was first found by the present inventors. The synthetic tissue is effective as a safe and efficient cell therapy system.

An object of the present invention is to provide a clinical application of the synthetic tissue regeneration of a joint tissue. The present invention provides the above-described synthetic tissue or a complex of a cell and a component derived from the cell, thereby making it possible to develop therapies for bone regeneration at a conventionally intractable site, in which both periosteum and bone cortex are inflamed; partial thickness cartilage injury which does not bleach the subchondral bone, and injury

of a meniscus, a tendon, a ligament, an intervertebral disk, cardiac muscle in an avascular area or a poor circulation site.

Thus, the present invention provides the following.

1. An implantable synthetic tissue.

2. A synthetic tissue according to item 1, which is biologically organized in the third dimensional direction.

3. A synthetic tissue according to item 1, which has biological integration capability with surroundings.

4. A synthetic tissue according to item 3, wherein the biological integration capability includes capability to adhere to surrounding cells and/or extracellular matrices.

5. A synthetic tissue according to item 1, which comprises cells.

6. A synthetic tissue according to item 1, which is substantially made of cells and a material derived from the cells.

7. A synthetic tissue according to item 1, which is substantially made of cells and an extracellular matrix (ECM) derived from the cells.

8. A synthetic tissue according to item 7, wherein the extracellular matrix contains at least one selected from the group consisting of collagen I, collagen III, vitronectin and fibronectin.

9. A synthetic tissue according to item 7, wherein the extracellular matrix contains collagen I, collagen III, vitronectin and fibronectin.

10. A synthetic tissue according to item 7, wherein the extracellular matrix contains vitronectin.

11. A synthetic tissue according to item 7, wherein the extracellular matrix contains fibronectin.

12. A synthetic tissue according to item 7, wherein the extracellular matrix contains collagen I and collagen III, the collagen constitutes 5% to 25% of the tissue, and the ratio of the collagen I to the collagen III is between 1:10 and 10:1.

13. A synthetic tissue according to item 7, wherein the extracellular matrix and the cells are integrated together into a three-dimensional structure.

14. A synthetic tissue according to item 7, wherein the extracellular matrix is diffusedly distributed in the tissue.

15. A synthetic tissue according to item 1, wherein an extracellular matrix is diffusedly distributed, and the distribution densities of the extracellular matrix in two arbitrary sections of 1 cm^2 in the tissue have a ratio within a range of about 1:3 to about 3:1.

16. A synthetic tissue according to item 1, which is heterologous, allogenic, isologous, or autogenous.

17. A synthetic tissue according to item 1, which is free of scaffolds.

18. A synthetic tissue according to item 1, which is used to implant cells.

19. A synthetic tissue according to item 1, which is large sized.

20. A synthetic tissue according to item 1, which has a volume of at least about 20 mm^3.

21. A synthetic tissue according to item 1, which is flexible.

22. A synthetic tissue according to item 1, which is expandable and contractile.

23. A synthetic tissue according to item 1, which can withstand heart pulsation.

24. A synthetic tissue according to item 1, which is biologically organized in all three dimensional directions.

25. A synthetic tissue according to item 24, wherein the biological integration is selected from the group consisting of internal binding of extracellular matrix, electrical integration, and intercellular signal transduction.

26. A synthetic tissue according to item 1, which has a tissue strength which allows the synthetic tissue to be clinically applicable.

27. A synthetic tissue according to item 26, wherein the strength is a break strength of about 0.02 N to about 2 N.

28. A synthetic tissue according to item 26, wherein the tissue strength is sufficient to provide self-supporting ability.

29. A synthetic tissue according to item 28, wherein the self-supporting ability is characterized in that the synthetic tissue is not substantially broken when the synthetic tissue is picked up using forceps having a tip area of 0.05 to 3.0 mm^2.

30. A synthetic tissue according to item 28, wherein the self-supporting ability is characterized in that the synthetic tissue is not broken when the synthetic tissue is picked up with a hand.

31. A synthetic tissue according to item 26, wherein the site to which the synthetic tissue is intended to be applied, includes a heart.

32. A synthetic tissue according to item 26, wherein the site to which the synthetic tissue is intended to be applied, includes an intervertebral disk, a meniscus, a cartilage, a bone, a ligament, or a tendon.

33. A synthetic tissue according to item 26, wherein:

the synthetic tissue is a cartilage, an intervertebral disk, a meniscus, a ligament, or a tendon; and

the synthetic tissue remains attached without an additional fixation procedure, after the synthetic tissue

is implanted into an injured portion of the intra-articular tissue.

34. A method for producing a synthetic tissue, comprising the steps of:

A) providing cells;

B) placing the cells in a container, the container having cell culture medium containing an ECM synthesis promoting agent and having a sufficient base area which can accommodate a synthetic tissue having a desired size;

C) culturing the cells in the container along with the cell culture medium containing the ECM synthesis promoting agent for a period of time suffificent for formation of the synthetic tissue having the desired size; and

D) detaching the cells from the container.

35. A method according to item 34, wherein a stimulus for inducing tissue contraction is applied in the detaching step.

36. A method according to item 35, wherein the stimulus includes a physical or chemical stimulus.

37. A method according to item 36, wherein the physical stimulus includes shaking of the container, pipetting, or deformation of the container.

38. A method according to item 34, wherein the detaching step includes adding an actin regulatory agent.

39. A method according to item 38, wherein the actin regulatory agent includes a chemical substance selected from the group consisting of actin depolymerizing agents and actin polymerizing agents.

40. A method according to item 39, wherein the actin depolymerizing agent is selected from the group consisting of Slingshot, cofilin, cyclase associated protein (CAP), actin interacting protein 1 (AIP1), actin depolymerizing factor (ADF), destrin, depactin, actophorin, cytochalasin, and NGF (nerve growth factor).

41. A method according to item 39, wherein the actin polymerizing agent is selected from the group consisting of RhoA, mDi, profilin, Rac1, IRSp53, WAVE2, ROCK, LIM kinase, cofilin, cdc42, N-WASP, Arp2/3, Drf3, Mena, lysophosphatidic acid (LPA), insulin, platelet derived growth factor (PDGF) a, PDGFb, chemokine, and transforming growth factor (TGF) β.

42. A method according to item 34, wherein the container is free of scaffolds.

43. A method according to item 34, wherein the cells are first cultured in monolayer culture.

44. A method according to item 34, wherein the ECM synthesis promoting agent includes TGFβ1, TFGβ3, ascorbic acid, ascorbic acid 2-phosphate, or a derivative or salt thereof.

45. A method according to item 44, wherein the ascorbic acid, ascorbic acid 2-phosphate, or the derivative or salt thereof is present at a concentration of at least 0.1 mM.

46. A method according to item 44, wherein the TGFβ1 or TFGβ3 is present at a concentration of at least 1 ng/ml.

47. A method according to item 34, wherein the cells are placed at a concentration of 5×10^4 to 5×10^6 cells per 1 cm^2, and the ECM synthesis promoting agent is ascorbic acid, ascorbic acid 2-phosphate, or a derivative or salt thereof, and the ascorbic acid, ascorbic acid 2-phosphate, or the derivative or salt thereof is provided at a concentration of at least 0.1 mM.

48. A method according to item 34, further comprising causing the synthetic tissue to detach from the container and self-contract.

49. A method according to item 48, wherein the detaching and self-contraction are achieved by providing a physical stimulus to the container.

50. A method according to item 48, wherein the detachment and self-contraction are achieved by providing a chemical stimulus to the container.

51. A method according to item 34, wherein the sufficient period of time is at least 3 days.

52. A method according to item 34, wherein the sufficient period of time is at least 3 days and a period of time required for the synthetic tissue to be spontaneously detached from the container at a maximum.

53. A method according to item 52, wherein the period of time required for the synthetic tissue to be spontaneously detached from the container is at least 40 days.

54. A method according to item 34, further comprising:

causing the synthetic tissue to differentiate.

55. A method according to item 54, wherein the differentiation includes osteogenesis, chondrogenesis, adipogenesis, tendon differentiation, and ligament differentiaion.

56. A method according to item 55, wherein the osteogenesis is performed in medium containing dexamethasone, β-glycerophosphate, and ascorbic acid 2-phosphate.

57. A method according to item 56, wherein the medium contains at least one selected from the group consisting of BMP (bone morphogenetic protein)-2, BMP-4, and BMP-7.

58. A method according to item 55, wherein the chondrogenesis is performed in medium containing pyrubic acid, dexamethasone, ascorbic acid 2-phosphate, insulin, transferrin, and selenious acid.

59. A method according to item 58, wherein the medium contains at least one selected from the group consisting of BMP-2, BMP-4, BMP-7, TGF(transforming frowth factor)-β1 and TGF-β3.

60. A method according to item 54, wherein the differentiation step is performed before or after the detaching step.

61. A method according to item 54, wherein the differentiation step is performed after the detaching step.

62. A method according to item 34, wherein the cell includes

cells of 3 or more passages.

63. A method according to item 34, wherein the cells include cells of 3 to 8 passages.

64. A method according to item 34, wherein the cells are provided at a cell density of 5.0×10^4 to 5.0×10^6 cells/cm^2.

65. A method according to item 34, wherein the cells include myoblasts.

66. A method according to item 34, wherein the cells include fat-derived cells.

67. A method according to item 34, wherein the cells include synovium-derived cells.

68. A method according to item 34, wherein the cells include mesenchymal stem cells.

69. A method according to item 68, wherein the mesenchymal stem cells are derived from an adipose tissue, a synovial membrane, a tendon, a bone, or a bone marrow.

70. A method according to item 34, further comprising:

producing a plurality of the synthetic tissues and attaching the plurality of the synthetic tissues together to be integrated.

71. A cell culture composition for producing a synthetic tissue from cells, comprising:

A) an element for maintaining the cells; and

B) an extracellular matrix synthesis promoting

agent.

72. A method according to item 68, wherein the ECM synthesis promoting agent includes TGFβ1, TFGβ3, ascorbic acid, ascorbic acid 2-phosphate, or a derivative or salt thereof.

73. A method according to item 72, wherein TGFβ1 or TFGβ3 is present at a concentration of at least 1 ng/ml, or ascorbic acid, ascorbic acid 2-phosphate, or the derivative or salt thereof is present at a concentration of at least 0.1 mM.

74. A complex for reinforcing a portion of an organism, comprising cells and a component derived from the cells.

75. A complex according to item 74, which has biological integration capability with surroundings.

76. A complex according to item 75, wherein the biological integration capability include capability to adhere to surrounding cells and/or extracellular matrices.

77. A complex according to item 74, which is substantially made of cells and a material derived from the cells.

78. A complex according to item 74, which is substantially made of cells and an extracellular matrix derived from the cells.

79. A synthetic tissue according to item 78, wherein the extracellular matrix is selected from the group consisting of collagen I, collagen III, vitronectin and fibronectin.

80. A complex according to item 78, wherein the extracellular

matrix and the cells are integrated together into a three-dimensional structure.

81. A complex according to item 78, wherein the extracellular matrix is provided on a surface of the complex.

82. A complex according to item 78, wherein the extracellular matrix is diffusedly distributed on a surface of the complex.

83. A complex according to item 74, wherein an extracellular matrix is diffusedly distributed on a surface of the complex, and the distribution densities of the extracellular matrix in two arbitrary sections of 1 cm^2 in the complex have a ratio within a range of about 1:3 to about 3:1.

84. A complex according to item 78, wherein the extracellular matrix includes fibronectin or vitronectin.

85. A complex according to item 74, which is heterologous, allogenic, isologous, or autogenous.

86. A complex according to item 74, wherein the portion includes a bag-shaped organ.

87. A complex according to item 86, wherein the bag-shaped organ includes a heart.

88. A complex according to item 74, wherein the portion includes a bone or cartilage tissue.

89. A complex according to item 74, wherein the portion includes avascular tissue.

90. A complex according to item 74, wherein the portion includes an intervertebral disk, a meniscus, a ligament, or a tendon.

91. A complex according to item 74, wherein the reinforcement is achieved by replacing the portion with the complex or providing the complex to cover the portion, or both.

92. A complex according to item 74, which resists the expansion and contraction of the portion.

93. A complex according to item 74, which has biological integration.

94. A complex according to item 74, wherein the biological integration selected from the group consisting of internal binding of extracellular matrix, electrical integration, and intercellular signal transduction.

95. A complex according to item 74, which is formed by culturing cells in the presence of an ECM synthesis promoting agent.

96. A complex according to item 74, which has self-supporting ability.

97. A method for reinforcing a portion of an organism, comprising the steps of:

A) replacing the portion with a complex comprising cells and a component derived from the cells or providing the complex to cover the portion, or both; and

B) holding the complex for a sufficient period of time for biologically adhering the complex to the portion.

98. A method according to item 97, wherein the adhesion is achieved by adhesion between extracellular matrix and extracellular matrix.

99. A method according to item 97, which has biological integration capability with surroundings.

100. A method according to item 99, wherein the biological integration capability include capability to adhere to surrounding cells and/or extracellular matrices.

101. A method according to item 97, which is substantially made of cells and a material derived from the cells.

102. A method according to item 97, which is substantially made of cells and an extracellular matrix derived from the cells.

103. A method according to item 102, wherein the extracellular matrix contains one selected from the group consisting of collagen I, collagen III, vitronectin and fibronectin.

104. A method according to item 102, wherein the extracellular matrix contains all of collagen I, collagen III, vitronectin and fibronectin.

105. A method according to item 102, wherein the extracellular matrix contains vitronectin.

106. A method according to item 102, wherein the extracellular matrix contains fibronectin.

107. A method according to item 97, wherein an extracellular matrix is provided on a surface of the complex.

108. A method according to item 97, wherein an extracellular matrix is diffusedly distributed on a surface of the complex.

109. A method according to item 97, wherein an extracellular matrix is diffusedly distributed on a surface of the complex, and the distribution densities of the extracellular matrix in two arbitrary sections of 1 cm^2 have a ratio within a range of about 1:3 to about 3:1.

110. A complex according to item 97, wherein an extracellular matrix is diffusedly distributed on a surface of the complex, and the distribution densities of the extracellular matrix in two arbitrary sections of 1 cm^2 have a ratio within a range of about 1:2 to about 2:1.

111. A method according to item 97, which is heterologous, allogenic, isologous, or autogenous.

112. A method according to item 97, wherein the portion includes a bag-shaped organ.

113. A method according to item 112, wherein the bag-shaped organ includes a heart.

114. A method according to item 97, wherein the complex resists the expansion and contraction of the portion.

115. A method according to item 97, wherein the complex has biological integration.

116. A method according to item 115, wherein the biological integration selected from the group consisting of internal binding of extracellular matrix, electrical integration, and intercellular signal transduction.

117. A method according to item 97, further comprising:

forming the complex by culturing the cells in the presence of an ECM synthesis promoting agent.

118. A method according to item 97, wherein the portion is a heart and the heart has a disease or disorder selected from the group consisting of heart failure, ischemic heart disease, myocardial infarct, cardiomyopathy, myocarditis, hypertrophic cardiomyopathy, dilated phase hypertrophic cardiomyopathy, and dilated cardiomyopathy.

119. A method according to item 97, wherein the portion includes an avascular lesion.

120. A method according to item 97, wherein the portion includes a vascular lesion.

121. A method according to item 97, wherein the portion includes a bone or a cartilage.

122. A method according to item 97, wherein the portion includes an intervertebral disk, a meniscus, a ligament, or a tendon.

123. A method according to item 97, wherein the portion includes a bone or a cartilage, and the bone or the cartilage is damaged or degenerated.

124. A method according to item 97, wherein the portion includes intractable fracture, osteonecrosis, cartilage injury, meniscus injury, ligament injury, tendon injury, cartilage degeneration, meniscus degeneration, intervertebral disk denaturation, ligament degeneration, or tendon degeneration.

125. A method according to item 97, wherein the sufficient period of time is at least 10 days.

126. A method according to item 97, wherein the complex has self-supporting ability.

127. A method according to item 97, which has biological integration capability with surroundings.

128. A method according to item 97, which is substantially made of cells and an extracellular matrix derived from the cells.

129. A method according to item 97, further comprising implanting another synthetic tissue.

130. A method according to item 129, wherein the other synthetic tissue is an artificial bone or a microfibrous collagen medical device.

131. A method according to item 97, which is substantially made of cells and an extracellular matrix derived from the cells, wherein the other synthetic tissue is an artificial bone or a microfibrous collagen medical device.

132. A method according to item 130, the artificial bone includes hydroxyapatite.

133. A method for treating a portion of an organism, comprising the steps of:

A) replacing the portion with a complex comprising cells and a component derived from the cells or providing the complex to cover the portion, or both; and

B) holding the complex for a sufficient period of time for restoring a condition of the portion.

134. A method according to item 133, wherein the treatment is for the treatment, prevention, or reinforcement of a disease, disorder, or condition of a heart, a bone, a cartilage, a ligament, a tendon, a meniscus, or an intervertebral disk.

135. A method according to item 133, wherein the complex has self-supporting ability.

136. A method according to item 133, wherein the complex has biological integration capability with surroundings.

137. A method according to item 133, wherein the complex is substantially made of cells and an extracellular matrix derived from the cells.

138. A method according to item 133, further comprising implanting another synthetic tissue in addition to the replacement or coverage of the portion.

139. A method according to item 138, wherein the other synthetic tissue includes an artificial bone or a

microfibrous collagen medical device.

140. A method according to item 133, which is substantially made of cells and an extracellular matrix derived from the cells, wherein the other synthetic tissue includes an artificial bone or a microfibrous collagen medical device.

141. A method according to item 139, the artificial bone includes hydroxyapatite.

142. A method for producing a synthetic tissue, comprising the steps of:

A) providing cells;

B) placing the cells in a container, the container having cell culture medium containing an ECM synthesis promoting agent and having a sufficient base area which can accommodate a synthetic tissue having a desired size;

C) culturing the cells in the container along with the cell culture medium containing the ECM synthesis promoting agent for a period of time suffificent for formation of the synthetic tissue having the desired size; and

D) regulating a thickness of the synthetic tissue by a physical or chemical stimulus to a desired thickness.

143. A method according to item 142, wherein the physical stimulus includes shear stress between the synthetic tissue and the container, deformation of the base of the container, shaking of the container, or pipetting.

144. A method according to item 142, wherein the chemical stimulus is obtained by using a chemical substance selected from the group consisting of actin depolymerizing agents and actin polymerizing agents.

145. A method according to item 144, wherein the actin depolymerizing agent is selected from the group consisting of Slingshot, cofilin, CAP (cyclase associated protein), AIP1 (actin interacting protein 1), ADF (actin depolymerizing factor), destrin, depactin, actophorin, cytochalasin, and NGF (nerve growth factor).

146. A method according to item 144, wherein the actin polymerizing agent is selected from the group consisting of RhoA, mDi, profilin, Rac1, IRSp53, WAVE2, ROCK, LIM kinase, cofilin, cdc42, N-WASP, Arp2/3, Drf3, Mena, LPA (lysophosphatidic acid), insulin, PDGF (platelet derived growth factor), PDGFb, chemokine, and TGF (transforming growth factor) β.

147. A method according to item 144, wherein the desired thickness is regulated by adjusting a ratio of the actin depolymerizing agent to the actin polymerizing agent.

148. A method according to item 142, further comprising:

producing a plurality of the synthetic tissues and attaching the plurality of the synthetic tissues together to be integrated.

149. A tissue complex, comprising an implantable synthetic tissue and another synthetic tissue.

150. A tissue complex according to item 149, wherein the implantable synthetic tissue is substantially made of cells and a material derived from the cells.

151. A tissue complex according to item 149, wherein the

implantable synthetic tissue is substantially made of cells and an extracellular matrix derived from the cells.

152. A tissue complex according to item 151, wherein the extracellular matrix is selected from the group consisting of collagen I, collagen III, vitronectin, and fibronectin.

153. A tissue complex according to item 151, wherein the extracellular matrix contains all of collagen I, collagen III, vitronectin, and fibronectin.

154. A tissue complex according to item 149, wherein the other synthetic tissue includes an artificial bone or a microfibrous collagen medical device.

155. A tissue complex according to item 154, the artificial bone includes hydroxyapatite.

156. A tissue complex according to item 149, the implantable synthetic tissue is biologically integrated with the other synthetic tissue.

157. A tissue complex according to item 156, wherein the biological integration is achieved via an extracellular matrix.

158. A composition for use in producing a synthetic tissue having a desired thickness, comprising a chemical substance selected from the group consisting of actin depolymerizing agents and actin polymerizing agents.

159. A composition according to item 158, wherein the actin depolymerizing agent is selected from the group consisting

of Slingshot, cofilin, CAP (cyclase associated protein), AIP1 (actin interacting protein 1), ADF (actin depolymerizing factor), destrin, depactin, actophorin, cytochalasin, and NGF (nerve growth factor).

160. A composition according to item 158, wherein the actin polymerizing agent is selected from the group consisting of RhoA, mDi, profilin, Rac1, IRSp53, WAVE2, ROCK, LIM kinase, cofilin, cdc42, N-WASP, Arp2/3, Drf3, Mena, LPA (lysophosphatidic acid), insulin, PDGF (platelet derived growth factor) a, PDGFb, chemokine, and TGF (transforming growth factor) β.

Hereinafter, the present invention will be described by way of preferable examples. It will be understood by those skilled in the art that the examples of the present invention can be appropriately made or carried out based on the description of the present specification and commonly used techniques well known in the art. The function and effect of the present invention can be easily recognized by those skilled in the art.

The present invention provides a scaffold-free synthetic tissue or complex. By providing such a scaffold-free synthetic tissue, a therapeutic method and a therapeutic agent for providing an excellent therapeutic result after implantation can be obtained.

The scaffold-free synthetic tissue of the present invention solves a long outstanding problem with biological formulations, which is attributed to contamination of the scaffold itself. Despite the absence of a scaffold, the therapeutic effect is comparable with, or more satisfactory

than conventional techniques.

In addition, when a scaffold is used, the alignment of implanted cells in the scaffold, the cell-to-cell adhesion, the in vivo alteration of the scaffold itself (eliciting inflammation), the integration of the scaffold to recipient tissue, and the like become problematic. These problems can be solved by the present invention.

The synthetic tissue and the complex of the present invention are also self-organized, and have biological integration inside thereof. Also on this point, the present invention is distinguished from conventional cell therapies.

It is easy to form a three-dimensional structure with the synthetic tissue or complex of the present invention, and thus it is easy to design it into a desired form. The versatility of the synthetic tissue and the complex of the present invention should be noted.

The synthetic tissue and the complex of the present invention have biological integration with recipient tissues, such as adjacent tissues, cells, and the like. Therefore, the post-operational stability is satisfactory, and cells are securely supplied to a local site, for example. An effect of the present invention is that the satisfactory biological integration capability allows the formation of a tissue complex with another synthetic tissue or the like, resulting in a complicated therapy.

Another effect of the present invention is that differentiation can be induced after the synthetic tissue or the complex is provided. Alternatively, differentiation

is induced before providing a synthetic tissue and/or a complex, and thereafter, the synthetic tissue and/or the complex are developed.

Another effect of the present invention is that the implantation of the synthetic tissue of the present invention provides a satisfactory tissue replacement ability and a comprehensive supply of cells for filling or covering an implanted site, compared to conventional cell-only implantation and sheet implantation.

The present invention provides an implantable synthetic tissue with biological integration capability. The above-described features and effects of the present invention make it possible to treat a site which cannot be considered as an implantation site for conventional synthetic products. The synthetic tissue of the present invention has biological integration and actually works in implantation therpies. The synthetic tissue is for the first time provided by the present invention, but is not provided by conventional techniques. The synthetic tissue or composite of the present invention has the sufficient ability to integrating with adjacent tissues, cells or the like during implantation (preferably, due to extracellular matrix). Therefore, post-operational restoration is excellent. Such a synthetic tissue, which has biological integration capability in all of the three dimensions, cannot be achieved by conventional techniques. Therefore, the present invention provides a therapeutic effect which cannot be achieved by conventional synthetic tissue.

In addition, the present invention provides medical treatment which provides a therapeutic effect by filling,

replacing, and/or covering a lesion.

In addition, when the synthetic tissue of the present invention is used in combination with another synthetic tissue (e.g., an artificial bone made of hydroxyapatite, a microfibrous collagen medical device, etc.), the synthetic tissue of the present invention is biologically integrated with the other synthetic tissue, so that the acceptance of the synthetic tissue makes it possible to organize more complicated tissue complex which is not conventionally expected.

An extracellular matrix or a cell adhesion molecule, such as fibronectin, vitronectin, or the like, is distributed throughout the synthetic tissue of the present invention. In the cell sheet engineering, a cell adhesion molecule is localized on a bottom surface of culture cells which is attached to a Petri dish. In the sheet provided by the cell sheet engineering, cells are major components of the sheet. The sheet is intended to provide a mass of cells with an adhesion molecule attached on the bottom surface. The synthetic tissue of the present invention is a real "tissue" such that an extracellular matrix three-dimensionally integrates with cells. Thus, the present invention is significantly distinguished from conventional techniques including the cell sheet engineering.

A cell implanting method without a scaffold has been reported by a Tokyo Women's Medical University group, in which a cell sheet is produced using a temperature sensitive culture dish. Such a cell sheet engineering technique is internationally appraised due to its originality. However, a single sheet obtained by this technique is fragile. In

order to obtain the strength that can withstand surgical manipulation, such as implantation, a plurality of sheets need to be piled up, for example. Such a problem is solved by the present invention.

A cell/matrix complex developed by the present invention does not require a temperature sensitive culture dish unlike the cell sheet technique. It is easy for the cell/matrix complex to form into a contractile three-dimensional tissue. There is no technique in the world other than the present invention, which can produce a contractile three-dimensional complex having 10 or more layers of cells without using so-called feeder cells, such as rodent stroma cells, in about three weeks. By adjusting conditions for matrix synthesis of the cell, it is possible to produce a complex having a strength which allows surgical manipulation, such as holding or transferring the complex, without a special instrument. Therefore, the present invention is an original, epoch-making technique in the world for reliably and safely perform cell implantation.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure **1** shows macroscopy and histology of exemplary synthetic tissues using synovial cells.

Figure **2** shows high magnification histology of a synthetic tissue when ascorbic acid 2-phosphate has a concentration of 0 mM, 0.1 mM, 1 mM, and 5 mM. As can be seen, Eosin staining of the synthetic tissue is more intense when ascorbic acid 2-phosphate is added at a concentration of more than 0.1 mM.

Figure **3** shows a high magnification view of a synthetic tissue on day 3, 7, 14, and 21 of culture. As can be seen, the synthetic tissue is already developed at day 3 but the matrix is scarce. The matrix is getting dense with time.

Figure **4** shows an exemplary stained extracellular matrix in a synthetic tissue derived from synovial cells.

Figure **5** shows exemplary histology of normal tissue (normal skin tissue, synovial membrane tissue, tendon tissue, cartilage tissue, and meniscus tissue).

Figure **6** shows exemplary histology of a commercially available stained collagen sponge as a control. From the left, staining of fibronectin, vitronectin, non-IgG-immune as a negative control and HE staining are shown.

Figure **7** shows the results of collagen content measurement. When 0.1 mM or more of ascorbic acid diphosphate is added, collagene content in the synthetic tissue of the present invention is significantly increased in any of the culture periods. However, substantially no difference among the concentrations of 0.1 mM, 1 mM and 5 mM were found.

Figure **8** shows the results of collagen content measurement. When 0.1 mM or more of ascorbic acid diphosphate is added, collagene content in the synthetic tissue of the present invention is significantly increased in any of the culture periods. However, substantially no difference among the concentrations of 0.1 mM, 1 mM and 5 mM were found.

Figure **9** shows a production of synthetic tissues

using a different number of cells. P represents the number of passages. Numeral figures in the photograph indicate the number of cells per cm^2.

Figure **10** shows a production of synthetic tissues using dishes with different sizes. * indicates culture in a 35-mm dish. ** indicates culture in a 60-mm dish. *** indicates culture in a 100-mm dish.

Figure **11** shows an exemplary mechanical testing system for measuring mechanical properties.

Figure **12** shows a test piece holding portion of an apparatus for measuring mechanical properties.

Figure **13** shows an enlarged view of an apparatus for measuring mechanical properties. A test piece is provided with a marker.

Figure **14** shows an enlarged view of a test piece holding portion.

Figure **15** shows a disrupted synthetic tissue after a tensile test.

Figure **16** shows the results (load-deformation curve) of a tensile test of a synthetic tissue (derived from synovium) of the present invention.

Figure **17** shows the results (stress-strain curve) of a mechanical properties test of a synthetic tissue (derived from synovial membrane tissue) of the present invention.

Figure **18** shows an exemplary osteogenic induction experiment of the synthetic tissue of the present invention and the results. The upper half portion shows a scheme for osteogenesis induction. The induction was conducted in the presence of 0.1 μM dexamethasone, 10 mM β-glycerophosphate, and 50 μg/ml ascorbic acid 2-phosphate. The lower left portion shows a control. The middle left portion shows a synthetic tissue differentiated into a bone by osteogenic induction. The middle lane portion shows Alizarin Red staining. The lower right portion shows an ALP-stained control. The middle right portion shows positive ALP-staining in a synthetic tissue by osteogenic induction.

Figure **19** shows the results of chondrogenic differentiation of a synthetic tissue of the present invention. This figure shows cultured synthetic tissues (A) and monolayer (B) using, from the leftmost, normal culture medium, chondrogenic medium, chondrogenic medium plus BPM-2 and chondrogenic medium plus TGF-β1, respectively. Note that A) synthetic tissues have more intense staining of Alcian blue than B) monolayer culture. Also, note that addition of TGF-β results in detachment of a synthetic tissue from the container without mechanical stimulation. (A) Most right lane.

Figure **20** shows semi-quantification of Alcian blue staining for comparison of a synthetic tissue of the present invention with a single cell sheet under chondrogenic stimulation as in Figures **19** and **39**. The left (blue) shows a result of monolayer, and the right (red) shows a result of the synthetic tissue.

Figure **21** shows the expression of various

chondrogenic marker genes (aggrecan, Col II, Sox9, B-actin) under chondrogenic stimulation.

Figure **22** shows the comparison of the expression of chondrogenic marker genes within a synthetic tissue and a monolayer culture of synovial cells under chondrogenic stimulation as in Figures **19** and **21**.

Figure **23** shows an *in vitro* cartilage implantation experiment using a synthetic tissue of the present invention and the results. The upper portion shows a diagram of explant culture. It is shown that a synthetic tissue is adhered to a partial thickness cartilage injury (*in vitro*). A superficial zone was removed, followed by digestion with chondroitinase ABC (Hinziker EB, JBJS, 1996). The lower left portion is lower magnification histology (×40). The lower right portion is higher magnification histology (×200). As can be seen, the synthetic tissue is tightly attached to the injured surface.

Figure **24** shows an *in vivo* cartilage implantation experiment of the present invention and the 10 day results. A synthetic tissue is firmly adhered to a partial cartilage injury. The left shows a macroscopic view of the result. The upper right shows a histology (×40) and the lower right shows a histology at higher magnification (×200).

Figure **25** shows the adhesion of a synthetic tissue of the present invention in a cartilage implantation experiment. The state on day 10 is shown. The left portion shows the result of HE staining, the middle portion shows the result of fibronectin staining, and the right portion shows the result of vitronectin staining.

Figure **26** shows the 1-month result of an in vivo implantation experiment of the present invention. A synthetic tissue is integrated with adjacent cartilage tissue without inflammation. Further, a superficial portion of the synthetic tissue contained a number of fibroblast-like cells (Figure **27**), and a deep portion of the synthetic tissue conatined a number of chondrocyte-like cells (Figure **28**), indicating the chondrogenesis of the synthetic tissue after the implantation at particularly deep portions.

Figure **27** shows a superficial portion of a synthetic tissue at one month after implantation.

Figure **28** shows a deep portion of a synthetic tissue at one month after implantation.

Figure **29** shows the result of a meniscus repair experiment using a synthetic tissue of the present invention. The left portion of the figure shows that a medial femoral condyle bone and an anterior horn of medial meniscus are exposed. The right figure shows a 6.5-mm defect in a medial knee joint in the anterior horn of medial meniscus.

Figure **30** shows a meniscus repair procedure. The left portion shows a defect before the implantation of a synovial membrane-derived synthetic tissue (lower left). The right portion shows the defect after the implantation of the synovial membrane-derived synthetic tissue.

Figure **31** shows the results of a meniscus repair experiment using a synthetic tissue of the present invention. A visual inspection four weeks after operation is shown.

The upper portion shows a state of a cartilage. It is shown that substantially no degeneration or injury due to friction or the like was found on the corresponding chondral surface, i.e., the meniscal defect was recovered. The lower left and right portions show a repaired defect.

Figure **32** shows the results of a meniscus repair experiment using a synthetic tissue of the present invention. The upper portion shows a macroscopic view. The lower left portion shows histology of a repaired tissue. The lower right portion shows histology of a border between the repaired tissue and its adjacent meniscus (magnification: x200).

Figure **33** shows an immunohistochemistry of a synthetic tissue derived from adipose tissue. From the left, H&E staining, fibronectin staining, and vitronectin staining.

Figure **34** shows the results of osteogenic or chondrogenic induction of a synthetic tissue derived from adipose tissue.

Figure **35** shows the results of a synthetic tissue with osteogenic induction when dexamethasone and β-glycerophosphate were added in culture medium prior to a detachment procedure.

Figure **36** shows the results of a synthetic tissue with osteogenic induction when dexamethasone and β-glycerophosphate were added in culture medium after a detachment procedure.

Figure **37** shows histology of biological integration of collagen gel containing synovial cells with cartilage after implantation. There is failure in integration observed (arrow).

Figure **38** shows biological integration after implantation to a chondral defect when a synthetic tissue of the present invention was used. The biological integration is completely established.

Figure **39** shows the effect of TGF-β on the detachment of a synthetic tissue. Addition of TGF-β leads to active detachment of the synthetic tissue.

Figure **40** shows a transition in contraction of a synthetic tissue of the present invention where dihydrochytochalasin or Y27632 was added or not. Data is shown in predetermined culture time intervals.

Figure **41** shows a photograph indicating adhesion of a synthetic tissue of the present invention with an artificial bone after fourteen days of culture in chondrogenic medium.

Figure **42** shows histology of a synthetic tissue cultured on a collagen synthetic tissue (CMI collagen sponge, Amgen, USA), which is a microfibrous collagen medical device, for 7 days.

Figure **43** shows a skeletal muscle-derived sheet developed by a synthetic tissue production method without ascorbic acid.

Figure **44** shows a skeletal muscle-derived synthetic

tissue developed by a synthetic tissue production method with ascorbic acid according to the present invention.

Figure **45** shows histology of the synthetic tissue as shown in Figure **44** (HE staining).

Figure **46** shows a diagram for explaining a technique for measuring stress and distortion characteristics to determine tensile strength.

Figure **47** shows a principle for obtaining a load/removal of a load curve.

(Description of Sequencing List)

SEQ ID NO.: 1 indicates the nucleic acid sequence of myosin heavy chain IIa (human: Accession No. NM_017534).

SEQ ID NO.: 2 indicates the amino acid sequence of myosin heavy chain IIa (human: Accession No. NM_017534).

SEQ ID NO.: 3 indicates the nucleic acid sequence of myosin heavy chain IIb (human: Accession No. NM_017533).

SEQ ID NO.: 4 indicates the amino acid sequence of myosin heavy chain IIb (human: Accession No. NM_017533).

SEQ ID NO.: 5 indicates the nucleic acid sequence of myosin heavy chain IId (IIx) (human: Accession No. NM_005963).

SEQ ID NO.: 6 indicates the amino acid sequence of myosin heavy chain IId (IIx) (human: Accession

No. NM_005963).

SEQ ID NO.: 7 indicates the nucleic acid sequence of CD56 (human: Accession No. U63041).

SEQ ID NO.: 8 indicates the amino acid sequence of CD56 (human: Accession No. U63041).

SEQ ID NO.: 9 indicates the nucleic acid sequence of human MyoD (GENBANK Accession No. X56677).

SEQ ID NO.: 10 indicates a polypeptide sequence encoded by the nucleic acid sequence of SEQ ID NO.: 2.

SEQ ID NO.: 11 indicates the nucleic acid sequence of human myogenic factor 5 (MYF5) (GENBANK Accession No. NM_005593).

SEQ ID NO.: 12 indicates a polypeptide sequence encoded by the nucleic acid sequence of SEQ ID NO.: 3.

SEQ ID NO.: 13 indicates the nucleic acid sequence of human myogenin (myogenic factor 4) (GENBANK Accession No. BT007233).

SEQ ID NO.: 14 indicates a polypeptide sequence encoded by the nucleic acid sequence of SEQ ID NO.: 13.

SEQ ID NO.: 15 indicates the nucleic acid sequence of Sox9 (human : Accession No. NM_000346 = a marker specific to a chondrocyte).

SEQ ID NO.: 16 indicates a polypeptide sequence

encoded by the nucleic acid sequence of SEQ ID NO.: 15.

SEQ ID NO.: 17 indicates the nucleic acid sequence of Col 2A1 (human: Accession No. NM_001844 = a marker specific to a chondrocyte).

SEQ ID NO.: 18 indicates a polypeptide sequence encoded by the nucleic acid sequence of SEQ ID NO.: 17.

SEQ ID NO.: 19 indicates the nucleic acid sequence of Aggrecan (human:Accession No. NM_001135 = a marker specific to a chondrocyte).

SEQ ID NO.: 20 indicates a polypeptide sequence encoded by the nucleic acid sequence of SEQ ID NO.: 19.

SEQ ID NO.: 21 indicates the nucei acid sequenence of Bone sialoprotein (human: Accession No. NM_004967 = a marker specific to an osteoblast).

SEQ ID NO.: 22 indicates a polypeptide sequence encoded by the nucleic acid sequence of SEQ ID NO.: 21.

SEQ ID NO.: 23 indicates the nucleic acid sequence of Osteocalcin (human: Accession No. NM_199173 = a marker specific to an osteoblast).

SEQ ID NO.: 24 indicates a polypeptide sequence encoded by the nucleic acid sequence of SEQ ID NO.: 23.

SEQ ID NO.: 25 indicates the nucleic acid sequence of GDF5 (human: Accession No. NM_000557 = a marker specific to a ligament cell).

SEQ ID NO.: 26 indicates a polypeptide sequence encoded by the nucleic acid sequence of SEQ ID NO.: 25.

SEQ ID NO.: 27 indicatesthe nucleic acid sequence of Six1 (human: Accession No. NM_005982 = a marker specific to a ligament cell).

SEQ ID NO.: 28 indicatesa polypeptide sequence encoded by the nucleic acid sequence of SEQ ID NO.: 27.

SEQ ID NO.: 29 indicates the nucleic acid sequence of Scleraxis (human: Accession No. BK000280 = a marker specific to a ligament cell).

SEQ ID NO.: 30 indicatesa polypeptide sequence encoded by the nucleic acid sequence of SEQ ID NO.: 29.

BEST MODE FOR CARRYING OUT THE INVENTION

The present invention will be described below. It should be understood throughout the present specification that articles for singular forms include the concept of their plurality unless otherwise mentioned. Therefore, articles or adjectives for singular forms (e.g., "a", "an", "the", and the like in English) include the concept of their plurality unless otherwise specified. Also, it should be also understood that terms as used herein have definitions ordinarily used in the art unless otherwise mentioned. Therefore, all technical and scientific terms used herein have the same meanings as commonly understood by those skilled in the relevant art. Otherwise, the present application (including definitions) takes precedence.

(Definition of terms)

The definitions of specific terms used herein are described below.

(Regenerative medicine)

As used herein, the term "regeneration" refers to a phenomenon in which when an individual organism loses a portion of tissue, the remaining tissue grows and recovers. The extent or manner of regeneration varies depending among animal species or among tissues in the same individual. Most human tissues have limited regeneration capability, and therefore, complete regeneration is not expected if a large portion of tissue is lost. In the case of severe damage, a tissue may grow which has strong proliferation capability different from that of lost tissue, resulting in incomplete regeneration where the damaged tissue is incompletely regenerated and the function of the tissue cannot be recovered. In this case, a structure made of a bioabsorbable material is used to prevent a tissue having strong proliferation capability from infiltrating the injured portion of the tissue so as to secure space for proliferation of the damaged tissue. Further, by supplementing with a cell growth factor, the regeneration capability of the damaged tissue is enhanced. Such a regeneration technique is applied to cartilages, bones, hearts, and peripheral nerves, for example. It has been so far believed that cartilages, nerve cells, and cardiac muscles have no or poor regeneration capability. Recently, it was reported that there are tissue (somatic stem cells), which have both the capability of differentiating into these tissues and self-proliferation capability. Expectations are running high for regenerative medicine using stem cells. Embryonic stem cells (ES cells) also have the capability

of differentiating into all tissues. Efforts have been made to use ES cells for regeneration of complicated organs, such as kidney, liver, and the like, but have not yet been realized.

The term "cell" is herein used in its broadest sense in the art, referring to a structural unit of tissue of a multicellular organism, which is capable of self replicating, has genetic information and a mechanism for expressing it, and is surrounded by a membrane structure which isolates the living body from the outside. In the method of the present invention, any cell can be used as a subject. The number of cells used in the present invention can be counted through an optical microscope. When counting using an optical microscope, the number of nuclei is counted. Tissues are sliced into tissue sections, which are then stained with hematoxylin-eosin (HE) to variegate nuclei derived from extracellular matrices (e.g., elastin or collagen) and cells. These tissue sections are observed under an optical microscope and the number of nuclei in a particular area (e.g., 200 μm × 200 μm) can be estimated to be the number of cells. Cells used herein may be either naturally-occurring cells or artificially modified cells (e.g., fusion cells, genetically modified cells, etc.). Examples of cell sources include, but are not limited to, a single-cell culture; the embryo, blood of a normally-grown transgenic animal; a cell mixture of cells derived from normally-grown cell lines; and the like. Primary culture cells may be used. Alternatively, subcultrue cells may also be used. Preferably, when subculture cells are used, the cells are preferably of 3 to 8 passages. As used herein, cell density may be represented by the number of cells per unit area (e.g., cm^2).

As used herein, the term "stem cell" refers to a cell capable of self replication and pluripotency. Typically, stem cells can regenerate an injured tissue. Stem cells used herein may be, but are not limited to, embryonic stem (ES) cells or tissue stem cells (also called tissular stem cell, tissue-specific stem cell, or somatic stem cell). A stem cell may be an artificially produced cell (e.g., fusion cells, reprogrammed cells, or the like used herein) as long as it can have the above-described abilities. Embryonic stem cells are pluripotent stem cells derived from early embryos. An embryonic stem cell was first established in 1981, and has been applied to production of knockout mice since 1989. In 1998, a human embryonic stem cell was established, which is currently becoming available for regenerative medicine. Tissue stem cells have a relatively limited level of differentiation unlike embryonic stem cells. Tissue stem cells are present in tissues and have an undifferentiated intracellular structure. Tissue stem cells have a higher nucleus/cytoplasm ratio and have few intracellular organelles. Most tissue stem cells have pluripotency, a long cell cycle, and proliferative ability beyond the life of the individual. As used herein, stem cells may be preferably embryonic stem cells, though tissue stem cells may also be employed depending on the circumstance.

Tissue stem cells are separated into categories of sites from which the cells are derived, such as the dermal system, the digestive system, the bone marrow system, the nervous system, and the like. Tissue stem cells in the dermal system include epidermal stem cells, hair follicle stem cells, and the like. Tissue stem cells in the digestive system include pancreatic (common) stem cells, hepatic stem cells, and the like. Tissue stem cells in the bone marrow system

include hematopoietic stem cells, mesenchymal stem cells, and the like. Tissue stem cells in the nervous system include neural stem cells, retinal stem cells, and the like.

As used herein, the term "somatic cell" refers to any cell other than a germ cell, such as an egg, a sperm, or the like, which does not transfer its DNA to the next generation. Typically, somatic cells have limited or no pluripotency. Somatic cells used herein may be naturally-occurring or genetically modified as long as they can achieve the intended treatment.

The origin of a stem cell is categorized into the ectoderm, endoderm, or mesoderm. Stem cells of ectodermal origin are mostly present in the brain, including neural stem cells. Stem cells of endodermal origin are mostly present in bone marrow, including blood vessel stem cells, hematopoietic stem cells, mesenchymal stem cells, and the like. Stem cells of mesoderm origin are mostly present in organs, including hepatic stem cells, pancreatic stem cells, and the like. As used herein, somatic cells may be derived from any mesenchyme. Preferably, somatic cells derived from mesenchyme may be employed.

As cells for use in construction of a synthetic tissue or three-dimensional structure of the present invention, differentiated cells or stem cells derived from the above-described ectoderm, endoderm, or mesoderm may be employed, for example. Examples of such cells include mesenchymal cells. In a certain embodiment, as such cells, myoblasts (e.g., skeletal myoblast, etc.), fibroblasts, synovial cells, and the like may be employed. As such cells, differentiated cells or stem cells can be used as they are.

Cells differentiated from stem cells into a desired direction can be used.

As used herein, the term "mesenchymal stem cell" refers to a stem cell found in mesenchyme. The term "mesenchymal stem cell" may be herein abbreviated as "MSC". Mesenchyme refers to a population of free cells which are in the asterodal shape or have irregular projections and bridge gaps between epithelial tissues, and which are recognized in each stage of development of multicellular animals. Mesenchyme also refers to tissue formed with intracellular cement associated with the cells. Mesenchymal stem cells have proliferation ability and the ability to differentiate into osteocytes, chondrocytes, muscle cells, stroma cells, tendon cells, and adipocytes. Mesenchymal stem cells are employed in order to culture or grow bone marrow cells or the like collected from patients, or differentiate them into chondrocytes or osteoblasts. Mesenchymal stem cells are also employed as reconstruction material, such as alveolar bones; bones, cartilages or joints for arthropathy or the like; and the like. There is a large demand for mesenchymal stem cells. A synthetic tissue or three-dimensional structure of the present invention comprising mesenchymal stem cells or differentiated mesenchymal stem cells is particularly useful when a structure is required in these applications.

As used herein, the term "isolated" means that naturally accompanying material is at least reduced, or preferably substantially completely eliminated, in normal circumstances. Therefore, the term "isolated cell" refers to a cell substantially free of other accompanying substances (e.g., other cells, proteins, nucleic acids, etc.) in natural

circumstances. The term "isolated tissue" refers to a tissue substantially free of substances other than that tissue (e.g., in the case of synthetic tissues or complexs, substances, scaffolds, sheets, coats, etc. used when the synthetic tissue is produced). As used herein, the term "isolated" refers to a scaffold-free state. Therefore, it will be understood that the synthetic tissue or complex of the present invention in the isolated state may contain components (e.g., medium, etc.) used in the production of it. The term "isolated" in relation to nucleic acids or polypeptides means that, for example, the nucleic acids or the polypeptides are substantially free of cellular substances or culture media when they are produced by recombinant DNA techniques; or precursory chemical substances or other chemical substances when they are chemically synthesized. Isolated nucleic acids are preferably free of sequences naturally flanking the nucleic acid within an organism from which the nucleic acid is derived (i.e., sequences positioned at the 5′ terminus and the 3′ terminus of the nucleic acid).

As used herein, the term "scaffold-free" indicates that a synthetic tissue does not substantially contain a material (scaffold) which is conventionally used for production of a synthetic tissue. Examples of such a scaffold include, but are not limited to, chemical polymeric compounds, ceramics, or biological formulations such as polysaccharides, collagens, gelatins, hyaluronic acids, and the like. A scaffold is a material which is substantially solid and has a strength which allows it to support cells or tissue.

As used herein, the term "established" in relation to cells refers to a state of a cell in which a particular

property (pluripotency) of the cell is maintained and the cell undergoes stable proliferation under culture conditions. Therefore, established stem cells maintain pluripotency.

As used herein, the term "non-embryonic" refers to not being directly derived from early embryos. Therefore, the term "non-embryonic" refers to cells derived from parts of the body other than early embryos. Also, modified embryonic stem cells (e.g., genetically modified or fusion embryonic stem cells, etc.) are encompassed by non-embryonic cells.

As used herein, the term "differentiated cell" refers to a cell having a specialized function and form (e.g., muscle cells, neurons, etc.). Unlike stem cells, differentiated cells have no or little pluripotency. Examples of differentiated cells include epidermic cells, pancreatic parenchymal cells, pancreatic duct cells, hepatic cells, blood cells, cardiac muscle cells, skeletal muscle cells, osteoblasts, skeletal myoblasts, neurons, vascular endothelial cells, pigment cells, smooth muscle cells, adipocytes, osteocytes, chondrocytes, and the like.

As used herein, the term "tissue" refers to a group of cells having the same function and form in cellular organisms. In multicellular organisms, constituent cells are usually differentiated so that the cells have specialized functions, resulting in division of labor. Therefore, multicellular organisms are not simple cell aggregations, but constitute organic or social cell groups having a certain function and structure. Examples of tissues include, but are not limited to, integument tissue, connective tissue, muscular tissue, nervous tissue, and the like. Tissue

targeted by the present invention may be derived from any organ or part of an organism. In a preferable embodiment of the present invention, tissue targeted by the present invention includes, but is not limited to, a bones, a cartilage, a tendon, a ligament, a meniscus, an intervertebral disk, a periosteum, a blood vessel, a blood vessel-like tissue, a heart, a cardiac valve, a pericardium, a dura mater, and the like.

As used herein, the term "cell sheet" refers to a structure comprising a monolayer of cells. Such a cell sheet has at least a two-dimensional biological integration. The sheet having biological integration is characterized in that after the sheet is produced, the connection between cells is not substantially destroyed even when the sheet is handled singly. Such biological integration includes intracellular connection via an extracellular matrix. It will be understood that the cell sheet may partially include a two or three-layer structure.

As used herein, the term "synthetic tissue" refers to tissue having a state different from natural states. Typically, a synthetic tissue is herein prepared by cell culture. Tissue which is removed from an organism and is not subjected to any treatment is not referred to as a synthetic tissue. Therefore, a synthetic tissue may include materials derived from organisms and materials not derived from organisms. The synthetic tissue of the present invention typically comprises a cell and/or a biological material, and may comprise other materials. More preferably, a synthetic tissue of the present invention is composed substantially only of a cell and/or a biological material. Such a biological material is preferably derived from cells

constituting the tissue (e.g., extracellular matrix, etc.).

As used herein, the term "implantable synthetic tissue" refers to a synthetic tissue, which can be used for actual clinical implantation and can function as a tissue at the implantation site for a certain period of time after implantation. Implantable synthetic tissue typically has sufficient biocompatibility, sufficient affinity, and the like.

The sufficient strength of an implantable synthetic tissue varies depending on a part targeted by implantation, but can be determined as appropriate by those skilled in the art. The strength is sufficient to provide self-supporting ability, and can be determined depending on the environment of implantation. The strength can be measured by measuring stress or distortion characteristics or conducting s creep characteristics indentation test as described below. The strength may also be evaluated by observing the maximum load.

The sufficient size of an implantable synthetic tissue varies depending on a part targeted by implantation, but can be determined as appropriate by those skilled in the art. The size can be determined depending on the environment of implantation.

However, an implantable synthetic tissue preferably has at least a certain size. Such a size (e.g., area) is at least 1 cm^2, preferably at least 2 cm^2, more preferably at least 3 cm^2, even more preferably at least 4 cm^2, at least 5 cm^2, at least 6 cm^2, at least 7 cm^2, at least 8 cm^2, at least 9 cm^2, at least 10 cm^2, at least 15 cm^2, or at least 20 cm^2.

An essence of the present invention is that a synthetic tissue of any size (area, volume) can be produced, i.e., the size is not particularly limited.

When the size is represented by the volume, the size may be, but is not limited to, at least 2 mm^3, or at least 40 mm^3. The size may be 2 mm^3 or less or 40 mm^3 or more.

The sufficient thickness of an implantable synthetic tissue varies depending on a part targeted by implantation, but can be determined as appropriate by those skilled in the art. The thickness can be determined depending on the environment of implantation. The thickness may exceed 5 mm. When an implantable synthetic tissue is implanted into the heart, the tissue may only have these minimum thicknesses. When implantable synthetic tissue is used in other applications, the tissue may preferably have a greater thickness. In such a case, for example, an implantable synthetic tissue has preferably a thickness of at least 2 mm, more preferably at least 3 mm, and even more preferably 5 mm. For example, when an implantable synthetic tissue is applied to a bone, a cartilage, a ligament, a tendon, or the like, similar to the case of a heart, the tissue has a thickness of at least about 1 mm (e.g., at least 2 mm, more preferably at least 3 mm, and even more preferably 5 mm), or 5 mm or more or less than 1 mm. An essence of the present invention is that a synthetic tissue or complex of any thickness can be produced, i.e., the size is not particularly limited.

The sufficient biocompatibility of implantable synthetic tissue varies depending on a part targeted by implantation, but can be determined as appropriate by those skilled in the art. However, an implantable synthetic tissue

preferably has at least a certain level of biocompatibility. Typically, a desired level of biocompatibility is, for example, such that biological integration to surrounding tissues is achieved without any inflammation, any immune reaction or the like. The present invention is not limited to this. In some cases (e.g., corneas, etc.), an immune reaction is less likely to occur. Therefore, an implantable synthetic tissue has biocompatibility to an extent, which achieves the object of the present invention even when an immune reaction is likely to occur in other organs. Examples of parameters indicating biocompatibility include, but are not limited to, the presence or absence of an extracellular matrix, the presence or absence of an immune reaction, the degree of inflammation, and the like. Such biocompatibility can be determined by examining the compatibility of a synthetic tissue at an implantation site after implantation (e.g., confirming that an implanted synthetic tissue is not destroyed). See "Hito Ishoku Zoki Kyozetsu Hanno no Byori Soshiki Shindan Kijyun Kanbetsu Shindan to Seiken Hyohon no Toriatsukai (Zufu) Jinzo Ishoku, Kanzo Ishoku Oyobi Shinzo Ishoku [Pathological Tissue Diagnosis Criterion for Human Transplanted Organ Rejection Reaction Handling of Differential Diagnosis and Biopsy Specimen (Illustrated Book) Kidney Transplantation, Liver Transplantation and Heart Transplantation]" The Japan Society for Transplantation and The Japanese Society for Pathology editors, Kanehara Shuppan Kabushiki Kaisha (1998). According to this document, biocompatibility is divided into Grade 0, 1A, 1B, 2, 3A, 3B, and 4. At Grade 0 (no acute rejection), no acute rejection reaction, cardiomyocyte failure, or the like is found in biopsy specimens. At Grade 1A (focal, mild acute rejection), there is focal infiltration of large lymphocytes around blood vessels or into

interstitial tissue, while there is no damage to cardiomyocytes. This observation is obtained in one or a plurality of biopsy specimens. At Grade 1B (diffuse, mild acute rejection), there is diffuse infilitration of large lymphocytes around blood vessels or into interstitial tissue or both, while there is no damage to cardiomyocytes. At Grade 2 (focal, moderate acute rejection), there is a single observed infilitration focus of inflammatory cells clearly bordered from the surrounding portions. Inflammation cells are large activated lymphocytes and may include eosinophils. Damage to cardiomyocytes associated with modification of cardiac muscle is observed in lesions. At Grade 3A (multifocal, moderate acute rejection), there are multiple infiltration foci of inflammatory cells which are large activated lymphocytes and may include eosinophils. Two or more of the multiple inflammatory infiltration foci of inflammatory cells have damages to cardiomyocytes. In some cases, there is also rough infiltration of inflammatory cells into the endocardium. The infiltration foci are observed in one or a plurality of biopsy specimens. At Grade 3B (multifocal, borderline severe acute rejection), there are more confluent and diffuse infiltration foci of inflammatory cells found in more biopsy specimens than those observed at Grade 3A. There is infiltration of inflammatory cells including large lymphocytes and eosinophils, in some cases neutrophils, as well as damage to cardiomyocytes. There is no hemorrhage. At Grade 4 (severe acute rejection), there is infiltration of various inflammatory cells including activated lymphocytes, eosinophils, and neutrophils. There is always damage to cardiomyocytes and necrosis of cardiomyocytes. Edema, hemorrhage, and/or angitis are also typically observed. Infiltration of inflammatory cells into the endocardium, which is different from the "Quilty"

effect, is typically observed. When a therapy is strongly conducted using an immunosuppressant for a considerably long period of time, edema and hemorrhage may be more significant than infilltration.

The sufficient affinity of an implantable synthetic tissue varies depending on a part targeted by implantation, but can be determined as appropriate by those skilled in the art. Examples of parameters for affinity include, but are not limited to, biological integration capability between an implanted synthetic tissue and its implantation site, and the like. Such affinity can be determined based on the presence of biological integration at an implantation site after implantation. Preferable affinity is herein such that an implanted synthetic tissue has the same function as that of a site in which the tissue is implanted, for example.

As used herein, the term "self-supporting ability" in relation to a tissue (e.g., a synthetic tissue, etc.) refers to a property of the synthetic tissue such that when it is restrained on at least one point thereof, it is not substantially destroyed. Self-supporting ability is herein observed if a tissue (e.g., a synthetic tissue) is picked up by using forceps with a tip having a thickness of 0.5 to 3.0 mm (preferably, forceps with a tip having a thickness of 1 to 2 mm or 1 mm; the forceps preferably have a bent tip) and the tissue is not substantially destroyed. Such forceps are commercially available (e.g., from Natsume Seisakusho, etc.). A force exerted for picking up a tissue is comparable with a force typically exerted by a medical practioner handing a tissue. Therefore, the self-supporting ability of a tissue can also be represented by a property such that the tissue is not destroyed when

it is picked up by a hand. Such forceps are, for example, but are not limited to, a pair of curved fine forceps (e.g., No. A-11 (tip: 1.0 mm in thickness) and No. A-12-2 (tip: 0.5 mm in thickness) commercially available from Natsume Seisakusho). A bent tip is suitable for picking up a synthetic tissue. The forceps are not limited to a bent tip type.

When a joint is treated, replacement is majorly performed. The strength of a synthetic tissue of the present invention required in such a case is such that a minimum self-supporting ability is obtained. Cells contained in the synthetic tissue are subsequently replaced with cells in an affected portion. The replacing cells produce a matrix which enhances the mechanical strength, so that the joint is healed. It will also be understood that the present invention may be used in conjunction with an artificial joint.

In the present invention, self-supporting ability plays an important role in evaluating the supporting ability of a synthetic tissue which is actually produced. When a synthetic tissue of the present invention is produced, the synthetic tissue is formed in the shape of a cell sheet in a container. Thereafter, the sheet is detached. With conventional techniques, the sheet is usually destroyed due to lack of self-supporting ability. Therefore, in conventional technology, an implantable synthetic tissue cannot be substantially produced. Especially, when a large-sized synthetic tissue is required, conventional techniques are not adequate. According to the technique of the present invention, a synthetic tissue can be produced, which has a sufficient strength which allows the tissue to be detached from a container without being destroying, i.e.,

the tissue already has self-supporting ability when being detached. This is true even when the synthetic tissue is in the form of a monolayer sheet before being detached. It will be understood that the monolayer may partially include a two or three-layer structure. Thus, it will be understood that the synthetic tissue of the present invention is applicable in substantially any chosen therapy. In addition, typically, after a synthetic tissue is produced and detached, the strength and self-supporting ability of the synthetic tissue are increased as observed in the present invention. Therefore, in the present invention, it will be understood that the self-supporting ability evaluated upon production may be an important aspect. In the present invention, the strength upon implantation is also important. It may also be important to evaluate the self-supporting ability of a synthetic tissue when a predetermined time has passed after the production of the tissue. Therefore, it will be understood that the self supporting ability at the time of implantation after transport, can be determined by calculating the time that has elapsed since production of the tissue, based on the above-described relationship.

As used herein, the term "membranous tissue" refers to a tissue in the form of membrane and is also referred to as "planar tissue". Examples of membranous tissue include tissues of organs (e.g., periosteum, pericardium, dura mater, cornea, etc.).

As used herein, the term "organ" refers to a structure which is a specific part of an individual organism where a certain function of the individual organism is locally performed and which is morphologically independent. Generally, in multicellular organisms (e.g., animals and

plants), organs are made of several tissues in specific spatial arrangement and tissue is made of a number of cells. Examples of such organs include, but are not limited to, skin, blood vessel, cornea, kidney, heart, liver, umbilical cord, intestine, nerve, lung, placenta, pancreas, brain, joint, bone, cartilage, peripheral limbs, retina, and the like. Examples of such organs include, but are not limited to, organs of the skin system, the parenchyma pancreas system, the pancreatic duct system, the hepatic system, the blood system, the myocardial system, the skeletal muscle system, the osteoblast system, the skeletal myoblast system, the nervous system, the blood vessel endothelial system, the pigment system, the smooth muscle system, the fat system, the bone system, the cartilage system, and the like.

As used herein, the term "bag-shaped organ" refers to an organ which has a three-dimensional expanse and the inside of which may be connected via a tubular tissue to the outside. Examples of bag-shaped organs include, but are not limited to, heart, liver, kidney, stomach, spleen, and the like.

In one embodiment, the present invention targets an intervertebral disk, a cartilage, a joint, a bone, a meniscus, a synovial membrane, a ligament, a tendon, and the like. In a preferable embodiment, the present invention targets blood vessels, blood vessel-like tissue, heart, heart valves, pericardia, dura mater, cornea, and bones. In another preferable embodiment, examples of organs targeted by the present invention include, but are not limited to, skeletal muscle, fat, and the like in addition to what is described above.

As used herein, the term "cover" or "wrap" in relation to a synthetic tissue, a three-dimensional structure, or the like, which is wrapped around a certain part (e.g., an injured site, etc.), means that the synthetic tissue or the like is arranged so as to cover the part (i.e., conceal an injury or the like). The terms "wrap" and "arrange (or locate) so as to cover" are used interchangeably. By observing the spatial relationship between the part and the synthetic tissue or the like, it can be determined whether or not the part is covered by the synthetic tissue or the like. In a preferable embodiment, in a covering step, a synthetic tissue or the like can be wrapped one turn around a certain site.

As used herein, the term "replace" means that a lesion (a site of an organism) is replaced, and cells which have originally been in a lesion are replaced with cells supplied by a synthetic tissue or a complex according to the present invention. Examples of a disease for which replacement is suitable include, but not limited to, a ruptured site, and the like. The term "fill" may be used in place of the term "replace" in the present specification.

A "sufficient time required for a synthetic tissue to biologically integrate with a part" herein varies depending on a combination of the part and the synthetic tissue, but can be determined as appropriate by those skilled in the art based on the combination. Examples of such a time include, but are not limited to, 1 week, 2 weeks, 1 month, 2 months, 3 months, 6 months, 1 year, and the like, after operation. In the present invention, a synthetic tissue preferably comprises substantially only cells and materials derived from the cells, and therefore, there is no particular

material which needs to be extracted after operation. Therefore, the lower limit of the sufficient time is not particularly important. Thus, in this case, a longer time is more preferable. If the time is substantially extremely long, reinforcement is substantially completed.

As used herein, the term "immune reaction" refers to a reaction due to the dysfunction of immunological tolerance between a graft and a host. Examples of immune reactions include, but are not limited to, a hyperacute rejection reaction (within several minutes after implantation) (immune reaction caused by antibodies, such as β-Gal or the like), an acute rejection reaction (reaction caused by cellular immunity about 7 to 21 days after implantation), a chronic rejection reaction (rejection reaction caused by cellular immunity 3 or more months after operation), and the like.

As used herein, the elicitation of an immune reaction can be confirmed by pathological and histological examination of the type, number, or the like of infiltration of (immunological) cells into implanted tissue using staining (e.g., HE staining, etc.), immunological staining, or microscopic inspection of tissue sections.

As used herein, the term "calcification" refers to precipitation of calcareous substances in organisms.

"Calcification" *in vivo* can be determined herein by staining (e.g., Alizarin Red staining) and measuring calcium concentration. Specifically, implanted tissue is taken out; the tissue section is dissolved by acid treatment or the like; and the atomic absorption of the solution is measured

by a trace element quantifying device.

As used herein, the term "within organism(s) (or in organism(s))" or "*in vivo*" refers to the inner part of organism(s). In a specific context, "within organism(s)" refers to a position at which a subject tissue or organ is placed.

As used herein, "*in vitro*" indicates that a part of an organism is extracted or released outside the organism for various purposes of research (e.g., in a test tube). The term *in vitro* is in contrast to the term *in vivo*.

As used herein, the term "*ex vivo*" refers to a series of operations where target cells into which a gene will be introduced are extracted from a subject; a therapeutic gene is introduced *in vitro* into the cells; and the cells are returned into the same subject.

As used herein, the term "material derived from cell(s)" refers to any material originating from the cell(s), including, but not being limited to, materials constituting the cell(s), materials secreted by the cell(s), materials metabolized by the cell(s), and the like. Representative examples of materials derived from cells include, but are not limited to, extracellular matrices, hormones, cytokines, and the like. Materials derived from cells typically have substantially no adverse effect on the cells and their hosts. Therefore, when the material is contained in a synthetic tissue, a three-dimensional structure, or the like, the material typically has substantially no adverse effect on the synthetic tissue, three-dimensional structure, or the like.

As used herein, the term "extracellular matrix" (ECM) refers to a substance existing between somatic cells no matter whether the cells are epithelial cells or non-epithelial cells. Extracellular matrices are typically produced by cells, and therefore, are biological materials. Extracellular matrices are involved in supporting tissue as well as in internal environmental structure essential for survival of all somatic cells. Extracellular matrices are generally produced from connective tissue cells. Some extracellular matrices are secreted from cells possessing basal membrane, such as epithelial cells or endothelial cells. Extracellular matrices are roughly divided into fibrous components and matrices filling there between. Fibrous components include collagen fibers and elastic fibers. A basic component of matrices is a glycosaminoglycan (acidic mucopolysaccharide), most of which is bound to non-collagenous protein to form a polymer of a proteoglycan (acidic mucopolysaccharide-protein complex). In addition, matrices include glycoproteins, such as laminin of basal membrane, microfibrils around elastic fibers, fibers, fibronectins on cell surfaces, and the like. Particularly differentiated tissue has the same basic structure. For example, in hyaline cartilage, chondroblasts characteristically produce a large amount of cartilage matrices including proteoglycans. In bones, osteoblasts produce bone matrices which cause calcification. Herein, examples of typical extracellular matrix include, but not limited to, collagen I, collagen III, collagen V, elastin, vitronectin, fibronectin, proteoglycans (for example, decolin, byglican, fibromodulin, lumican, hyaluronic acid, etc.). Various types of extracellular matrix may be utilized in the present invention as long as cell adhesion is acheived.

In one embodiment of the present invention, the synthetic tissue, three-dimensional structure, or the like of the present invention may be advantageously similar to the composition of an extracellular matrix (e.g., elastin, collagen (e.g., Type I, Type III, Type IV, etc.), laminin, etc.) of a site of an organ for which implantation is intended. In the present invention, extracellular matrices include cell adhesion molecules. As used herein, the terms "cell adhesion molecule" and "adhesion molecule" are used interchangeably, referring to a molecule capable of mediating the joining of two or more cells (cell adhesion) or adhesion between a substrate and a cell. In general, cell adhesion molecules are divided into two groups: molecules involved in cell-cell adhesion (intercellular adhesion) (cell-cell adhesion molecules) and molecules involved in cell-extracellular matrix adhesion (cell-substrate adhesion) (cell-substrate adhesion molecules). A synthetic tissue or three-dimensional structure of the present invention typically comprises such a cell adhesion molecule. Therefore, cell adhesion molecules herein include a protein of a substrate and a protein of a cell (e.g., integrin, etc.) in cell-substrate adhesion. A molecule other than proteins falls within the concept of cell adhesion molecule as long as it can mediate cell adhesion.

It should be noted that the synthetic tissue or complex of the present invention comprises cells and a ■ material (natively) derived from the cell.■ Therefore, such materials including ECMs form a complicated composition containg collagen I, collagen III, collagen V, elastin, fibronectin, vitronectin, proteoglycans (for example, decolin, byglican, fibromodulin, lumican, hyaluronic acid,

etc.). Conventionally a synthetic tissue containg such cell-derived ingredients has not been provided. To obtain a synthetic tissue having such a composition is substantially impossible when an artificial material is used. Thus, a composition containing such ingredients (particularly , collagen I, collagen III and the like) is recognized to be a native composition.

More preferably, an extracellular matrix includes all the collagen (for example, Types I, Type III, etc.), vitronectin, and fibronectin. Especially, a synthetic tissue containing vitronectin and/or fibronectin has not been provided before. Therefore, the synthetic tissue and the complex according to the present invention are recognized to be new in this regard.

As used herein, the term "provided" or "distributed" in relation to an extracellular matrix and the synthetic tissue of the present invention indicates that the extracellular matrix is present in the synthetic tissue. It should be understood that such superficial provision can be visualized and observed by immunologically staining an extracellular matrix of interest.

As used herein, the term "in a diffused manner" or "diffusedly" in relation to the distribution of an extracellular matrix indicates that the extracellular matrix is not localized. Such distribution of an extracellular matrix has a ratio of the distribution densities of two arbitrary sections of 1 cm^2 within a range of typically about 1:10 to about 10:1, and representatively about 1:3 to about 3:1, and preferably about 1:2 to about 2:1, and more preferably about 1:1 (i.e., substantially evenly distributed over the

synthetic tissue. When an extracellular matrix is distributed on a surface of the synthetic tissue of the present invention, but not localized, the synthetic tissue of the present invention has biological integration capability evenly with respect to the surrounding. Therefore, the synthetic tissue of the present invention has an excellent effect of recovery after implantation.

For cell-cell adhesion, cadherin, a number of molecules belonging in an immunoglobulin superfamily (NCAML1, ICAM, fasciclin II, III, etc.), selectin, and the like are known, each of which is known to join cell membranes via a specific molecular reaction. Therefore, in one embodiment, the synthetic tissue, three-dimensional structure, or the like of the present invention preferably has substantially the same composition of cadherin, immunoglobulin superfamily molecules, or the like as that of a site for which implantation is intended.

Thus, various molecules are involved in cell adhesion and have different functions. Those skilled in the art can appropriately select a molecule to be contained in a synthetic tissue or three-dimensional structure of the present invention depending on the purpose. Techniques for cell adhesion are well known as described above and as described in, for example, "Saibogaimatorikkusu -Rinsho heno Oyo- [Extracellular matrix -Clinical Applications-], Medical Review.

It can be determined whether or not a certain molecule is a cell adhesion molecule, by an assay, such as biochemical quantification (an SDS-PAG method, a labeled-collagen method, etc.), immunological quantification (an enzyme antibody

method, a fluorescent antibody method, an immunohistological study, etc.), a PCR method, a hybridization method, or the like, in which a positive reaction is detected. Examples of such a cell adhesion molecule include, but are not limited to, collagen, integrin, fibronectin, laminin, vitronectin, fibrinogen, an immunoglobulin superfamily member (e.g., CD2, CD4, CD8, ICM1, ICAM2, VCAM1), selectin, cadherin, and the like. Most of these cell adhesion molecules transmit into a cell an auxiliary signal for cell activation due to intercellular interaction as well as cell adhesion. Therefore, an adhesion molecule for use in an implant of the present invention preferably transmits an auxiliary signal for cell activation into a cell. This is because cell activation can promote growth of cells originally present or aggregating in a tissue or organ at an injured site after application of an implant thereto. It can be determined whether or not such an auxiliary signal can be transmitted into a cell, by an assay, such as biochemical quantification (an SDS-PAG method, a labeled-collagen method, etc.), immunological quantification (an enzyme antibody method, a fluorescent antibody method, an immunohistological study, etc.), a PDR method, a hybridization method, or the like, in which a positive reaction is detected.

An example of a cell adhesion molecule is cadherin which is present in many cells capable of being fixed to tissue. Cadherin can be used in a preferable embodiment of the present invention. Examples of a cell adhesion molecule in cells of blood and the immune system which are not fixed to tissue, include, but are not limited to, immunoglobulin superfamily molecules (LFA-3, CD2, CD4, CD8, ICAM-1, ICAM2, VCAM1, etc.); integrin family molecules (LFA-1, Mac-1, gpIIbIIIa, p150, p95, VLA1, VLA2, VLA3, VLA4, VLA5, VLA6,

etc.); selectin family molecules (L-selectin, E-selectin, P-selectin, etc.), and the like. Therefore, such a molecule may be useful for treatment of a tissue or organ of blood and the immune system.

Nonfixed cells need to be adhered to a specific tissue in order to act on the tissue. In this case, it is believed that cell-cell adhesion is gradually enhanced via a first adhesion by a selectin molecule or the like which is constantly expressed and a second adhesion by a subsequently activated integrin molecule. Therefore, in the present invention, a cell adhesion molecule for mediating the first adhesion and another cell adhesion molecule for mediating the second adhesion may be used together.

As used herein, the term "actin regulatory agent" refers to a substance which interacts directly or indirectly with actin in cells to change the form or state of the actin. It should be understood that actin regulatory agents are categorized into two classes, actin depolymerizing agents and actin polymerizing agents, depending on the action on actin. Examples of actin depolymerizing agents include, but are not limited to, Slingshot, cofilin, CAP (cyclase associated protein), ADF (actin depolymerizing factor), destrin, depactin, actophorin, cytochalasin, NGF (nerve growth factor), and the like. Examples of actin polymerizing agents include, but are not limited to, RhoA, mDi, profilin, Rac1, IRSp 53, Wave2, profilin, ROCK, Lim kinase, cofilin, cdc42, N-WASP, Arp2/3, Drf3, IRSp53, Mena, LPA (lysophosphatidic acid), insulin, PDGF (platelet-derived growth factor) a, PDGFb, chemokine, TGF (transforming growth factor) b, and the like. The above-described actin regulatory agents include some substances which can be

identified by the following assay. Interaction of an actin regulatory agent with respect to actin is assayed as follows. Actin is visualized using an actin staining reagent (Molecular Probes, Texas Red-X phalloidin) or the like. By observing actin aggregation or cell outgrowth under a microscope, the presence of the interaction is determined by confirming the aggregation and reconstruction of actin and/or an increase in the cell outgrowth rate. The determination may be performed quantitatively or qualitatively. The above-described actin regulatory agents are used in the present invention so as to promote the detachment or a multilayer structure of the synthetic tissue. When an actin regulatory agent used in the present invention is derived from an organism, the organism may be a mammalian species, such as human, mouse, bovine, or the like.

The above-described agents involved in actin polymerization control actin polymerization in relation to Rho and the examples of the agents include the follwing (see, for example, "Saibokokkaku/Undo ga wakaru (Understanding of cytoskeleton/movement)", (Ed./Hiroaki Miki), Yodo-sha).

Actin polymerization (see Takenaka T et al. J.Cell Sci., 114: 1801-1809, 2001)

RhoA → mDi → profilin ⇒ actin polymerization

RhoA → ROCK/Rho → LIM kinase → phosphorylation of (suppression) ⇒ actin polymerization

Rac1 → IRSp53 → WAVE2 → profilin, Arp2/3 ⇒ actin polymerization

cdc42 → N-WASP → profilin, Arp2/3 ⇒ actin polymerization

cdc42 → Drf3 → IRSp53 → Mena ⇒ actin polymerization

(In the above descriptions, → indicates a singal transduction pathway such as phosphorylation. In the present invention any agent involved in such a pathway can be utilized.

Actin depolymerization

Slingshot → dephosphorization of cofilin (activation) ⇒ actin depolymerization

Actin depolymerization is controlled by a balance between phosphorylation by LIM kinase activity of cofilin and dephosphorization by Slingshot. As another agent for activating cofilin, CAP(cyclase-associated protein) and AIPI(actin-interacting-protein 1) are identified. It is recognized that any suitable agent can be used.

LPA (lysophosphatidic acid) of any chain length can be used.

Any chemokine can be used. However, examples of prefereable chemokine include interleukin 8, MIP-1, SDF-1 and the like.

Any TGFβ can be used. However, examples of preferable TGFβ include TGF-β1 and TGF-β3. TGF-β1 and TGF-β3 has an extracellular matrix generation promoting activity. Thus, in the present invention, TGF-β1 and TGF-β3 are used

with an attention.

As used herein, the term "tissue strength" refers to a parameter which indicates a function of a tissue or organ and a physical strength of the tissue or organ. Tissue strength can be generally determined by measuring tensile strength (e.g., break strength, modulus of rigidity, Young's modulus, etc.). Such a general tensile test is well known. By analyzing data obtained by a general tensile test, various data, such as break strength, modulus of rigidity, Young's modulus, and the like, can be obtained. These values can be herein used as indicators of tissue strength. Typically, tissue strength which allows clinical applications is herein required.

The tensile strength of a synthetic tissue, three-dimensional structure, or the like of the present invention can be determined by measuring the stress and distortion characteristics thereof. Briefly, a load is applied to a sample; the resultant distortion and the load are input to respective A/D converters (e.g., ELK-5000) (1 ch: distortion, 2 ch: load); the stress and distortion characteristics are measured to determine the tensile strength of the sample (Figure **46**). Tensile strength can also be determined by testing creep characteristics. A creep characteristics indentation test is conducted to investigate how a sample is extended over time while a constant load is applied to the sample. For small materials, thin materials, and the like, an indentation test is conducted using, for example, a triangular pyramid-shaped indenter with a tip having a radius of about 0.1 μm to about 1 μm. Initially, the indenter is pushed into a test piece so that a load is given to the test piece. When the indenter reaches from

several tens of nanometers to several micrometers deep in the test piece, the indenter is drawn off to remove the load. Figure **47** shows a load/removal of load curve obtained by the above-described test method. Rigidity, Young's modulus, or the like can be obtained based on the behavior of the load and the push depth derived from the curve.

The tensile strength of the synthetic tissue of the present invention may be low. The tensile strength becomes higher when the matrix concentration is increased, and becomes lower when the cell to matrix ratio is increased. The present invention is characterized in that the strength can be adjusted as necessary. The present invention is also characterized in that the strength can be high or low relative to that of a tissue to be implanted. Therefore, it is recognized that the strength can be set to comply with any desired site.

As used herein, the term "physiologically active substance" refers to a substance capable of acting on a cell or tissue. Physiologically active substances include cytokines and growth factors. A cellular physiologically active substance may be naturally-occurring or synthesized. Preferably, a cellular physiologically active substance is one that is produced by a cell or one that has a function similar thereto. As used herein, a cellular physiologically active substance may be in the form of a protein or a nucleic acid or in other forms. In actual practice, cellular physiologically active substances are typically proteins. In the present invention, a physiologically active substance may be used to promote the affinity of an implanted synthetic tissue of the present invention, for example.

The term "cytokine" is used herein in the broadest sense in the art and refers to a physiologically active substance which is produced from a cell and acts on the same or different cell. Cytokines are generally proteins or polypeptides having a function of controlling an immune response, regulating the endocrine system, regulating the nervous system, acting against a tumor, acting against a virus, regulating cell growth, regulating cell differentiation, or the like. Cytokines are herein in the form of a protein or a nucleic acid or in other forms. In actual practice, cytokines are typically proteins.

The terms "growth factor" or "cell growth factor" are used herein interchangeably and each refers to a substance which promotes or controls cell growth. Growth factors are also called "proliferation factors" or "development factors". Growth factors may be added to cell or tissue culture medium, substituting for serum macromolecules. It has been revealed that a number of growth factors have a function of controlling differentiation in addition to a function of promoting cell growth.

Examples of cytokines representatively include, but are not limited to, interleukins, chemokines, hematopoietic factors such as colony stimulating factors, a tumor necrosis factor, interferons, a platelet-derived growth factor (PDGF), an epidermal growth factor (EGF), a fibroblast growth factor (FGF), a hepatocyte growth factor (HGF), a vascular endothelial cell growth factor (VEGF), cardiotrophin, and the like, which have proliferative activity.

Cellular physiologically active substances, such as cytokines, growth factors, and the like, typically have

redundancy in function. Accordingly, reference herein to a particular cytokine or growth factor by one name or function also includes any other names or functions by which the factor is known to those of skill in the art, as long as the factor has the activity of a cellular physiologically active substance for use in the present invention. Cytokines or growth factors can be used in a therapeutic or pharmaceutical agent according to a preferable embodiment of the present invention as long as they have preferable activity as described herein.

Therefore, in one embodiment of the present invention, it was revealed that when such a cytokine or growth factor (e.g., BMP-2, etc.) is provided to an implantation site (e.g., an injured site of a cartilage, etc.) concomitantly with a synthetic tissue or three-dimensional structure of the present invention, the affinity of the synthetic tissue or three-dimensional structure and an improvement in the function of the implantation site are observed. Thus, the present invention also provides such a combined therapy.

As used herein, the term "differentiation" refers to a developmental process of the state of the complex parts of organisms, such as cells, tissues, or organs and a process in which a characteristic tissue or organ is formed. The term "differentiation" is mainly used in embryology, developmental biology, and the like. In organisms, various tissues and organs are formed from divisions of a fertilized ovum (a single cell) to an adult. At early developmental stages (i.e., before cell division or after insufficient cell division), each cell or cell group has no morphological or functional feature and is not much distinguishable. Such a state is referred to as "undifferentiated".

"Differentiation" may occur at the level of organs. A cell constituting an organ may develop into various cells or cell groups having different features. This phenomenon is also referred to as differentiation within an organ in the formation of the organ. Therefore, a synthetic tissue or three-dimensional structure of the present invention may comprise a tissue including differentiated cells.

When differentiation is required to produce a synthetic tissue of the present invention, the differentiation may be performed either before or after the organization of the cells.

As used herein, the terms "differentiation agent" and "differentiation promoting agent" are used interchangeably and refer to any agent which is known to promote differentiation of cells (e.g., chemical substances, temperature, etc.). Examples of such an agent include, but are not limited to, various environmental factors, such as temperature, humidity, pH, salt concentration, nutrients, metals, gas, organic solvent, pressure, chemical substances (e.g., steroids, antibiotics, etc.), and the like, or arbitrary combinations thereof. Representative examples of differentiation agents include, but are not limited to, cellular physiologically active substances. Representative examples of cellular physiologically active substances include, but are not limited to, DNA demethylating agents (e.g., 5-azacytidine, etc.), histone deacetylating agents (e.g., trichosanthin, etc.), intraneclear receptor ligands (e.g., retinoic acid (ATRA), vitamin D_3, T3, etc.), cell growth factors (e.g., activin, IGF-1, FGF, PDGF, TGF-β, BMP2/4, etc.), cytokines (e.g., LIF, IL-2, IL-6, etc.), hexamethylenebisacetoamides, dimethylacetoamides, dibutyl

cAMPs, dimethylsulfoxides, iododeoxyuridines, hydroxyl ureas, cytosine arabinosides, mitomycin C, sodium lactate, aphydicolin, fluorodeoxyuridine, polybren hexadimetrine bromide, selenium, and the like.

Specific examples of differentiation agents are described below. These differentiation agents may be used singly or in combination.

A) Cornea: epidermal growth factor (EGF);
B) Skin (keratinocyte): TGF-β, FGF-7 (KGF: keratinocyte growth factor), EGF;
C) Vascular endothelium: VEGF, FGF, angiopoietin;
D) Kidney: LIF, BMP, FGF, GDNF;
E) Heart: HGF, LIF, VEGF;
F) Liver: HGF, TGF-β, IL-6, EGF, VEGF;
G) Umbilical endothelium: VEGF;
H) Intestinal epithelium: EGF, IGF-I, HGF, KGF, TGF-β, IL-11;
I) Nerve: nerve growth factor (NGF), BDNF (brain-derived neurotrophic factor), GDNF (glial-derived neurotrophic factor), neurotrophin, IL-6, TGF-β, TNF;
J) Glia cell: TGF-β, TNF-α, EGF, LIF, IL-6;
K) Peripheral nerve cell: bFGF, LIF, TGF-β, IL-6, VEGF;
L) Lung (alveolar epithelium): TGF-β, IL-13, IL-1β, KGF, HGF;
M) Placenta: growth hormone (GH), IGF, prolactin, LIF, IL-1, activin A, EGF;
N) Pancreatic epithelium: growth hormone, prolactin;
O) Pancreatic Langerhans' cells: TGF-β, IGF, PDGF, EGF, TGF-β, TRH (thyroropin);
P) Synovial cell: FGF, TGF-β (particularly, TGF-β1, TGF-β3);
Q) Osteoblast: BMP (particularly, BMP-2, BMP-4, BMP-7), FGF;
R) Chondroblast: FGF, TGF-β (particularly, TGF-β1, TGF-β3), BMP (particularly, BMP-2, BMP-4, BMP-7), TNF-α, IGF;

S) Retinal cell: FGF, CNTF (cilliary neurotrophic factor);
T) Fat cell: insulin, IGF, LIF; and
U) Muscle cell: LIF, TNF-α, FGF.

As used herein, the term "osteogenesis" indicates that any cell is caused to differentiate into a osteocyte. It is known that osteogenesis is promoted in the presence of dexamethasone, β-glycerophosphate, and ascorbic acid 2-phosphate. An osteogenic agent (BMP, (particularly, BMP-2, BMP-4, BMP-7)) may be added to promote osteogenesis.

As used herein, the term "chondrogenesis" refers to differentiation of any cell into a chondrocyte. It is known that chondrogenesis is promoted in the presence of pyrubic acid, dexamethasone, ascorbic acid 2-phosphate, insulin, transferrine, and selenious acid. An bone morphogenetic protein (BMP, (particularly, BMP-2, BMP-4, BMP-7)), TGF-β (particularly, TGF-β1 and TGF-β3), FGF, TNF-α and the like may be added to promote chondrogenesis.

As used herein, the term "adipogenesis" refers to differentiation of any cell into an adipocyte. It is known that adipogenesis is promoted in the presence of insulin, IGF, LIF, and ascorbic acid 2-phosphate.

As used herein, the terms "implant", "graft", and "tissue graft" are used interchangeably, referring to homologous or heterologous tissue or a cell group, or an artificial material, which is inserted into a particular site of a body and thereafter forms a part of the body. Therefore, a synthetic tissue or three-dimensional structure of the present invention can be used as an implant. Examples of conventional grafts include, but are not limited to, organs or portions of organs, blood vessels, blood vessel-like

tissue, heart, cardiac valves, pericardia, dura matter, joint capsule, bone, cartilage, cornea, tooth, and the like. Therefore, grafts encompass any one of these which is inserted into an injured part so as to compensate for the lost portion. Grafts include, but are not limited to, autografts, allografts, and xenografts, which depend on the type of their donor.

As used herein, the term "autograft" (a tissue, a cell, an organ, etc.) refers to a graft (a tissue, a cell, an organ, etc.) which is implanted into the same individual from which the graft is derived. As used herein, the term "autograft" (a tissue, a cell, an organ, etc.) may encompass a graft from a genetically identical individual (e.g. an identical twin) in a broad sense. As used herein, the terms "autologous" and "derived from a subject" are used interchangeably. Therefore, the term "not derived from a subject" in relation to a graft indicates that the graft is not autologous (i.e., heterologous).

As used herein, the term "allograft (a tissue, a cell, an organ, etc.)" refers to a graft (a tissue, a cell, an organ, etc.) which is transplanted from a donor genetically different from, though of the same species, as the recipient. Since an allograft is genetically different from the recipient, the allograft (a tissue, a cell, an organ, etc.) may elicit an immune reaction in the recipient. Examples of such grafts (a tissue, a cell, an organ, etc.) include, but are not limited to, grafts derived from parents (a tissue, a cell, an organ, etc.). The synthetic tissue of the present invention can be an allograft, which has been demonstrated to have satisfactory therapeutic results. Attention should be paid to the synthetic tissue of the present invention.

As used herein, the term "xenograft" (a tissue, a cell, an organ, etc.) refers to a graft (a tissue, a cell, an organ, etc.) which is implanted from a different species. Therefore, for example, when a human is a recipient, a porcine-derived graft (a tissue, a cell, an organ, etc.) is called a xenograft (a tissue, a cell, an organ, etc.).

As used herein, "recipient" (acceptor) refers to an individual which receives a graft (a tissue, a cell, an organ, etc.) or implanted matter (a tissue, a cell, an organ, etc.) and is also called "host". In contrast, an individual providing a graft (a tissue, a cell, an organ, etc.) or implanted matter (a tissue, a cell, an organ, etc.) is called "donor" (provider).

With a synthetic tissue forming technique of the present invention, a synthetic tissue derived from any cell can be used. This is because a synthetic tissue (e.g., membranous tissues, organs, etc.) formed by the method of the present invention can exhibit a desired function while the tissue injury rate is maintained at a level which does not interfere with the therapy (i.e., a low level). Conventionally, tissues or organs are used as grafts without modification. In contrast to this, the present invention provides a tissue comprising three-dimensionally connected cells. Such a synthetic three-dimensional tissue cannot be achieved by conventional techniques, and therefore, constitutes one significant effect of the present invention.

As used herein, the term "subject" refers to an organism to which treatment of the present invention is applied and is also referred to as "patient". A patient or

subject may be preferably a human.

Cells optionally used in a synthetic tissue, three-dimensional structure, or tissue graft of the present invention may be derived from a syngeneic origin (self origin), an allogenic origin (non-self origin), or a heterologous origin. In view of rejection reactions, syngeneic cells are preferable. If rejection reactions do not raise problems, allogenic cells may be employed. Cells which elicit rejection reactions can be employed by optionally treating the cells in a manner that overcomes rejection reactions. Procedures for avoiding rejection reactions are known in the art (see, for example, "Shin Gekagaku Taikei, Dai 12 Kan, Zoki Ishoku (Shinzo Ishoku · Hai Ishoku Gijutsuteki, Rinriteki Seibi kara Jisshi ni Mukete [New Whole Surgery, Vol. 12, Organ Transplantation (Heart Transplantation · Lung Transplantation From Technical and Ethical Improvements to Practice)" (Revised 3rd ed.), Nakayama Shoten]. Examples of such methods include, but are not limited to, a method using immunosuppressants or steroidal drugs, and the like. For example, there are currently the following immunosuppressants for preventing rejection reactions: "cyclosporine" (SANDIMMUNE/NEORAL); "tacrolimus" (PROGRAF); "azathioprine" (IMURAN); "steroid hormone" (prednine, methylprednine); and "T-cell antibodies" (OKT3, ATG, etc.). A method which is used worldwide as a preventive immunosuppression therapy in many facilities, is the concurrent use of three drugs: cyclosporine, azathioprine, and steroid hormone. An immunosuppressant is desirably administered concurrently with a pharmaceutical agent of the present invention. The present invention is not limited to this. An immunosuppressant may be administered before or after a regeneration/therapeutic method of the present

invention as long as an immunosuppression effect can be achieved.

Cells used in the present invention may be derived from any organism (e.g., vertebrates and invertebrates). Preferably, cells derived from vertebrates are used. More preferably, cells derived from mammals (e.g., primates, rodents, etc.) are used. Even more preferably, cells derived from primates are used. Most preferably, cells derived from a human are used. Typically, cells from the same species as the host are preferably used.

Examples of an affected portion of a subject treated by a synthetic tissue of the present invention include, but are not limited to, the heart suffering from a heart disease (e.g., heart failure, ischemic heart diseases, myocardial infarct, cardiomyopathy, myocarditis, hypertrophic cardiomyopathy, dilated hypertrophic cardiomyopathy, and dilated cardiomyopathy); blood vessels in a pericardium patch, infarcted myocardium lower and upper limbs; a joint injury or denaturation; a cartilage injury or denaturation; osteonecrosis; meniscus injury or denaturation; intervertebral disk denaturation; ligament injury or denaturation; a fracture; implantation to a patient having a joint, cartilage, or bone having bone loss; an injured cornea; and the like.

Tissues targeted by the present invention may be any organ of an organism and may be derived from any organism. Examples of organisms targeted by the present invention include vertebrates and invertebrates. Preferably, organisms targeted by the present invention are mammals (e.g., primates, rodents, etc.). More preferably, organisms

targeted by the present invention are primates. Most preferably, organisms targeted by the present invention are humans.

As used herein, the term "flexibility" in relation to a synthetic tissue refers to an ability to resist physical stimuli from external environments (e.g., pressure). A synthetic tissue having flexibility is preferable when the implantation site moves or deforms autonomously or by external effects.

As used herein, the term "extendibility and contractibility" in relation to a synthetic tissue refers to an ability to resist extending or contracting stimuli from external environments (e.g., pulsation). A synthetic tissue having extendibility and contractibility is preferable when the implantation site is subjected to extending or contracting stimuli. Examples of implantation sites, which are subjected to extending or contracting stimuli, include, but are not limited to, heart, muscle, joint, cartilage, tendon, and the like. In one embodiment, extendibility and contractibility capable of withstanding the pulsation motion of the heart may be required.

As used herein, the term "part" or "portion" refers to any part or portion, tissue, cell, or organ in the body. Examples of such parts, tissues, cells, and organs include, but are not limited to, a portion which can be treated with skeletal myoblasts, fibroblasts, synovial cells, stem cells, and the like. A marker specific to a portion may be any parameter, such as a nucleic acid molecule (expression of mRNA), a protein, an extracellular matrix, a specific phenotype, a specific shape of a cell, or the like. Therefore,

markers which are not specified herein may be used to identify a synthetic tissue of the present invention as long as these markers can indicate cells derived from a portion. Representative examples of portions, but are not limited to, portions of the heart other than the adult myocardium, portions containing mesenchymal stem cells or cells derived therefrom, other tissues, other organs, myoblasts (e.g., skeletal myoblasts), fibroblasts, synovial cells, and the like.

For observing a cartilage tissue, following markers can be used as index.

Sox9 (human: Accession No. NM_000346) is a marker specific to a chondrocyte. The marker can be confirmed mainly by observing the presence of mRNA (Kulyk WM, Franklin JL, Hoffman LM. Sox9 expression during chondrogenesis in micromass cultures of embryonic limb mesenchyme. Exp Cell Res. 2000 Mar 15, 255(2):327-32.).

Col 2A1 (human: Accession No. NM_001844) is a marker specific to a chondrocyte. The marker can be confirmed mainly by observing the presence of mRNA (Kulyk WM, Franklin JL, Hoffman LM. Sox9 expression during chondrogenesis in micromass cultures of embryonic limb mesenchyme. Exp Cell Res. 2000 Mar 15;255(2):327-32.).

Aggrecan (human: Accession No. NM_001135) is a marker specific to a chondrocyte. The marker can be confirmed mainly by observing the presence of mRNA (Kulyk WM, Franklin JL, Hoffman LM. Sox9 expression during chondrogenesis in micromass cultures of embryonic limb mesenchyme. Exp Cell Res. 2000 Mar 15;255(2):327-32.).

Bone sialoprotein (human: Accession No. NM_004967) is a marker specific to an osteoblast. The marker can be confirmed mainly by observing the presence of mRNA (Haase HR, Ivanovski S, Waters MJ, Bartold PM. Growth hormone regulates osteogenic marker mRNA expression in human periodontal fibroblasts and alveolar bone-derived cells. J Periodontal Res. 2003 Aug;38(4):366-74.).

Osteocalcin (human: Accession No. NM_199173) is a marker specific to an osteoblast. The marker can be confirmed mainly by observing the presence of mRNA (Haase HR, Ivanovski S, Waters MJ, Bartold PM. Growth hormone regulates osteogenic marker mRNA expression in human periodontal fibroblasts and alveolar bone-derived cells. J Periodontal Res. 2003 Aug;38(4):366-74.).

GDF5 (human :Accession No. NM_000557) is a marker specific to a ligament cell. The marker can be confirmed mainly by observing the presence of mRNA (Wolfman NM, Hattersley G, Cox K, Celeste AJ, Nelson R, Yamaji N, Dube JL, DiBlasio-Smith E, Nove J, Song JJ, Wozney JM, Rosen V. Ectopic induction of tendon and ligament in rats by growth and differentiation factors 5, 6, and 7, members of the TGF-beta gene family. J Clin Invest. 1997 Jul 15;100(2):321-30.).

Six1 (human: Accession No. NM_005982) is a marker specific to a ligament cell (Dreyer SD, Naruse T, Morello R, Zabel B, Winterpacht A, Johnson RL, Lee B, Oberg KC. Lmx1b expression during joint and tendon formation: localization and evaluation of potential downstream targets. Gene Expr Patterns. 2004 Jul;4(4):397-405.). The marker can be

confirmed mainly by observing the presence of mRNA.

Scleraxis (human :Accession No. BK000280) is a marker specific to a ligament cell (Brent AE, Schweitzer R, Tabin CJ. A somitic compartment of tendon progenitors. Cell. 2003 Apr 18;113(2):235-48.). The marker can be confirmed mainly by observing the presence of mRNA.

A "part other than the myocardium of an adult" and a "part other than the heart of an adult" can be identified using markers characteristic to cells derived from the myocardium of an adult or the heart of an adult including skeletal myoblasts, fibroblasts, synovial cells, stem cells, or the like (hereinafter referred to as a "non-adult myocardial marker" or a "non-adult heart marker", respectively). If the marker is expressed by less than about 100%, preferably less than about 80%, more preferably less than about 50%, even more preferably less than about 25%, in some cases less than about 1%, the above-described parts can be identified. Examples of such markers include, but are not limited to, myosin heavy chain IIa, myosin heavy chain IIb, myosin heavy chain IId (IIx), CD56, MyoD, Myf5, myogenin, and the like. Therefore, non-adult myocardial markers which are not specified herein may be used to identify a synthetic tissue of the present invention as long as these markers can indicate cells derived from parts other than the myocardium of an adult. Also, non-adult heart markers which are not specified herein may be used to identify a synthetic tissue of the present invention as long as these markers can indicate cells derived from parts other than the heart of an adult.

Myosin heavy chain IIa (human: Accession

No. NM_017534; SEQ ID NOs. 1 and 2), myosin heavy chain IIb (human: Accession No. NM_017533; SEQ ID NOs. 3 and 4), and myosin heavy chain IId (IIx) (human: Accession No. NM_005963; SEQ ID NOs. 5 and 6) are markers specific to myoblasts (Havenith M.G., Visser R., Schrijvers-van Schendel J.M., Bosman F.T., "Muscle Fiber Typing in Routinely Processed Skeletal Muscle With Monoclonal Antibodies", Histochemistry, 1990; 93(5):497-499). These markers can be confirmed mainly by observing the presence of proteins. An antibody against myosin heavy chain IIa, myosin heavy chain IIb, and myosin heavy chain IId (IIx) is, for example, MY-32 available from Sigma. This antibody is specific to skeletal muscles and does not bind to myocardium (Webster C., Pavlath G.K., Parks D.R., Walsh F.S., Blau H.M., Exp. Cell. Res., 1988 Jan; 174(1):252-65; and Havenith M.G., Visser R., Schrijvers-van Schendel J.M., Bosman F.T., Muscle Fiber Typing in Routinely Processed Skeletal Muscle with Monoclonal Antibodies, Histochemistry, 1990, 93(5):497-499).

CD56 (human: Accession No. U63041; SEQ ID NOs. 7 and 8) is a marker specific to myoblasts. This marker can be confirmed mainly by observing the presence of mRNA.

MyoD (human: Accession No. X56677; SEQ ID NOs. 9 and 10) is a marker specific to myoblasts. This marker can be confirmed mainly by observing the presence of mRNA.

Myf5 (human: Accession No. NM_005593; SEQ ID NOs. 11 and 12) is a marker specific to myoblasts. This marker can be confirmed mainly by observing the presence of mRNA.

Myogenin (human: Accession No. BT007233; SEQ ID NOs. 13 and 14) is a marker specific to myoblasts. This

marker can be confirmed mainly by observing the presence of mRNA.

In other embodiments, other markers specific to other tissues can be utilized. Examples of such markers include, but are not limited to, Oct-3/4, SSEA-1, Rex-1, Otx2, and the like for embryonic stem cells; VE-cadherin, Flk-1, Tie-1, PECAM1, vWF, c-kit, CD34, Thy1, Sca-1, and the like for endothelial cells; skeletal muscle α actin in addition to the above-described markers for skeletal muscles; Nestin, Glu receptor, NMDA receptor, GFAP, neuregulin-1, and the like for nerve cells; c-kit, CD34, Thy1, Sca-1, GATA-1, GATA-2, FOG, and the like for hematopoietic cells.

As used herein, the term "derived" in relation to cells means that the cells are separated, isolated, or extracted from a cell mass, tissue, or organ in which the cells have been originally present, or that the cells are induced from stem cells.

As used herein, the term "applicable to heart" means that the heart applied has an ability to pulsate. A tissue applicable to heart has strength such that the tissue can withstand dilation and contraction of the pulsating heart. Here, applicability to the heart includes applicability to the myocardium. Applicability to heart may be determined by confirming that a recipient having an implanted graft survives.

As used herein, the term "three-dimensional structure" refers to an object which comprises cells having intracellular intergration or alignment and extends three-dimensionally, particularly matrices are oriented

three-dimensionally and cells are arranged three-dimensionally.

As used herein, the term "biological integration" in relation to the relationship between biological entities such as cells means that there is certain interaction between the biological entities. Examples of such interaction includes, but are not limited to, interaction via biological molecules (e.g., extracellular matrix), interaction via signal transduction, electrical interaction (electrical integration, such as synchronization of electrical signals or the like), and the like. Biological integration includes biological integration in a synthetic tissue and biological integration of a synthetic tissue with its surroundings (e.g., surrounding tissues and cells after implantation, etc.). In order to confirm interactions, an assay appropriate to a characteristic of the interaction is employed. In order to confirm physical interactions via biological molecules, the strength of a synthetic tissue, a three-dimensional structure, or the like is measured (e.g., a tensile test). In order to confirm interaction via signal transduction, gene expression or the like is investigated. In order to confirm electrical interactions, the electric potential of a synthetic tissue, a three-dimensional structure, or the like is measured to determine whether or not the electric potential is propagated with constant waves. In the present invention, biological integration is provided in all three dimensions. Preferably, there is biological integration substantially uniformly in all directions in a three-dimensional space. In another embodiment, the synthetic tissue, a three-dimensional structure, and the like, which has substantially uniform two-dimensional biological integration and slightly weaker biological

integration in the third dimension, may be employed. Biological integration via an extracellular matrix can be confirmed based on the degree of staining by staining the extracellular matrix. As a method for observing biological integration *in vivo*, there is an integration experiment using cartilage. In this experiment, a surface of the cartilage is removed and digested with chondroitinase ABC (Hunziker E.B. et al., J. Bone Joint Surg. Am., 1996 May; 78(5): 721-33). Thereafter, a tissue of interest is implanted onto a cut surface, followed by culturing for about 7 days. The subsequent integration is observed (Figure **23**). It will be understood that a capability to adhere to surrounding cells can be determined with the above-described cartilage experiment.

A synthetic tissue, three-dimensional structure, or the like of the present invention may be provided using known preparation methods, as a pharmaceutical product, or alternatively, as an animal drug, a quasi-drug, a marine drug, a cosmetic product, and the like.

Animals targeted by the present invention include any organism as long as it has organs (e.g., animals (e.g., vertebrates, invertebrate)). Preferably, the animal is a vertebrate (e.g., Myxiniformes, Petronyzoniformes, Chondrichthyes, Osteichthyes, amphibian, reptilian, avian, mammalian, etc.), more preferably mammalian (e.g., monotremata, marsupialia, edentate, dermoptera, chiroptera, carnivore, insectivore, proboscidea, perissodactyla, artiodactyla, tubulidentata, pholidota, sirenia, cetacean, primates, rodentia, lagomorpha, etc.). Illustrative examples of a subject include, but are not limited to, animals, such as cattle, pigs, horses, chickens, cats, dogs, and the

like. More preferably, primates (e.g., chimpanzee, Japanese monkey, human, etc.) are used. Most preferably, a human is used. This is because there is limitation to implantation therapies.

When the present invention is used as a pharmaceutical agent, it may further comprise a pharmaceutically acceptable carrier or the like. A pharmaceutically acceptable carrier contained in a medicament of the present invention includes any material known in the art.

Examples of such a pharmaceutically acceptable carrier include, but are not limited to, antioxidants, preservatives, colorants, flavoring agents, diluents, emulsifiers, suspending agents, solvents, fillers, bulking agents, buffers, delivery vehicles, agricultural or pharmaceutical adjuvants, and the like.

The amount of a pharmaceutical agent (e.g., a synthetic tissue, a pharmaceutical compound used in conjunction therewith, etc.) used in the treatment method of the present invention can be easily determined by those skilled in the art with reference to the purpose of use, a target disease (type, severity, and the like), the patient's age, weight, sex, and case history, the form or type of the cell, and the like. The frequency of the treatment method of the present invention applied to a subject (or patient) is also determined by those skilled in the art with respect to the purpose of use, target disease (type, severity, and the like), the patient's age, weight, sex, and case history, the progression of the therapy, and the like. Examples of the frequency include once per day to several months (e.g., once per week to once per month). Preferably, administration

is performed once per week to month with reference to the progression.

As used herein, the term "administer" in relation to a synthetic tissue, three-dimensional structure, or the like of the present invention or a pharmaceutical agent comprising it, means that they are administered singly or in combination with other therapeutic agents. A synthetic tissue of the present invention may be introduced into therapy sites (e.g., impaired heart, etc.) by the following methods, in the following forms, and in the following amounts. Examples of the introduction methods include, but are not limited to, direct attachment, suture after attachment, insertion, and the like. For example, a synthetic tissue and a three-dimensional structure of the present invention may be applied by the above-described methods to an impaired site of ischemic myocardial tissue caused by myocardial infarct, angina pectoris, or the like. Combinations may be administered either concomitantly (e.g., as an admixture), separately but simultaneously or concurrently; or sequentially. This includes presentations in which the combined agents are administered together as a therapeutic mixture, and also procedures in which the combined agents are administered separately but simultaneously (e.g., a synthetic tissue or the like is directly provided by operation, while other pharmaceutical agents are provided by intravenous injection). "Combination" administration further includes the separate administration of one of the compounds or agents given first, followed by the second.

As used herein, the term "reinforcement" means that the function of a targeted part of an organism is improved.

As used herein, the term "instructions" describe how to handle reagents, usage, a preparation method, a method of producing a synthetic tissue, a method of administering a medicament of the present invention, a method for diagnosis, or the like for persons who administer, or are administered, the medicament or the like or persons who diagnose or are diagnosed (e.g, physicians, patients, and the like). The instructions describe a statement indicating an appropriate method for administering a diagnostic, a medicament, or the like of the present invention. The instructions are prepared in accordance with a format defined by an authority of a country in which the present invention is practiced (e.g., Health, Labor and Welfare Ministry in Japan, Food and Drug Administration (FDA) in the U.S., and the like), explicitly describing that the instructions are approved by the authority. The instructions are so-called package insert and are typically provided in paper media. The instructions are not so limited and may be provided in the form of electronic media (e.g., web sites, electronic mails, and the like provided on the Internet).

As used herein, the term "extracellular matrix synthesis promoting agent" or "ECM synthesis promoting agent" refers to an agent which promotes the production of an extracellular matrix of a cell. In the present invention, when an ECM synthesis promoting agent is added to a cell sheet, an environment which promotes self-contraction of cells after a cell sheet is detached from a culture container. The sheet is biologically organized in three-dimensional directions. Examples of such an agent representatively include agents capable of promoting the secretion of an extracellular matrix (e.g., TGF-β1, TGF-β3, etc.). Examples of an ECM synthesis promoting agent representatively include,

but are not limited to, TGF-β1, TGF-β3, ascorbic acid, ascorbic acid 2-phosphate, or a derivative or salt thereof. Preferably, an ECM synthesis promoting agent may be preferably a component of an extracellular matrix of a part targeted by application and/or a component(s) capable of promoting the secretion of an extracellular matrix in an amount similar thereto. When an ECM synthesis promoting agent comprises a plurality of components, the components may be components of an extracellular matrix of a part targeted by application and/or components capable of promoting the secretion of an extracellular matrix in an amount similar thereto.

As used herein, the term "ascorbic acid or a derivative thereof" includes ascorbic acid and an analog thereto (e.g., ascorbic acid 2-phosphate, ascorbic acid 1-phosphate, etc.), and a salt thereof (e.g., sodium salt, magnesium salt, etc.). Ascorbic acid is preferably, but is not limited to, an L-isomer.

(Description of the Preferred Embodiments)

Hereinafter, preferable embodiments of the present invention will be described. The following embodiments are provided for a better understanding of the present invention and the scope of the present invention should not be limited to the following description. It will be clearly appreciated by those skilled in the art that variations and modifications can be made without departing from the scope of the present invention with reference to the specification.

In an aspect of the present invention, the synthetic tissue and complex of the present invention is free of injury caused by a protein degrading enzyme, such as,

representatively, dispase, trypsin, or the like, during culture. Therefore, the synthetic tissue and complex, which is detached from the base material, can be recovered as a cell mass holding proteins between cells (e.g., an extracellular matrix) and having a certain level of strength. The synthetic tissue and complex also retain intact functions, such as an intracellular linking manner, alignment, and the like. When typical protein degrading enzymes (e.g., trypsin, etc.) are used to detach the three-dimensional structure or synthetic tissue, substantially no cell-to-cell link or cell-to-extracellular matrix link are retained, so that cells are individually separated. Among these protein degrading enzymes, dispase destroys basement membrane-like proteins between cells and base materials substantially completely. In this case, however, the resultant three-dimensional structure or synthetic tissue has weak strength. In contrast, the three-dimensional structure or synthetic tissue of the present invention can both substantially completely retain each of the desmosome structure and the basement membrane-like protein, resulting in the above-described various effects.

In the method of the present invention, the period of time required for culture may be determined depending on the application of the synthetic tissue or three-dimensional structure. In order to detach and recover the cultured synthetic tissue or three-dimensional structure from the support material, the cultured synthetic tissue or three-dimensional structure is detached directly, or with macromolecular membrane being attached thereto. Note that the synthetic tissue or three-dimensional structure may be detached in culture medium in which cells have been cultured, or alternatively, in other isotonic solutions. Such

solutions may be selected depending on the purpose. When a monolayer cell sheet is prepared, examples of the macromolecular membrane, which is optionally attached to the cell sheet or three-dimensional structure, include, but are not limited to, hydrophilized polyvinylidene difluoride (PVDF), polypropylene, polyethylene, cellulose and derivatives thereof, chitin, chitosan, collagen, paper (e.g., Japan paper, etc.), urethane, net-like or stockinette-like macromolecular materials (e.g., spandex, etc.), and the like. When a net-like or stockinette-like macromolecular material is employed, the synthetic tissue or complex has a higher degree of freedom, so that the contraction/relaxation function thereof can be increased. A method for producing the synthetic tissue or three-dimensional structure comprising cells of the present invention is not particularly limited. For example, the synthetic tissue or three-dimensional structure of the present invention can be produced by utilizing the above-described cultured cell sheet attached to a macromolecular membrane.

In order to detach and recover the synthetic tissue or complex with a high yield from the cell culture support, the cell culture support is tapped or shaken, or the medium is stirred with a pipette. These procedures may be performed singly or in combination. In addition, the synthetic tissue or complex may be detached and recovered by deforming the base of the culture container or rinsing the container with isotonic solution or the like. By stretching the synthetic tissue or complex in a specific direction after being detached from the base material, the complex is provided with alignment. Stretching may be performed by using a tensile device (e.g., Tensilon, etc.), or simply forceps, or the like. A stretching method is not particularly limited. By providing alignment,

it is possible to confer directionality to the motion of the cell sheet or complex itself. Therefore, for example, it is possible to allow the synthetic tissue or complex to move in accordance with the motion of a specific organ. The synthetic tissue or complex can be efficiently applied to organs.

The thus-obtained synthetic tissue or complex cannot be obtained by conventional techniques.

The synthetic tissue and the complex according to the present invention includes an abundance of adhesion molecules such as extracellular matrix which may include collagen (types I, III, etc.), vironectin, and fibronectin, and can be accepted by the surrounding tissue. Thus, implanted cells can be stably accepted by the implantation site. In conventional cell implantation, it was difficult for cells to be stably accepted by the implantation site not only in cells implantation without a scaffold, but also in cell implantation using an additional stabilizing treatment (e.g., sewing of a patch, scaffold, etc.). However, use of the present invention facilitates stabilization. When only cells are used, reinforcement by another tissue, fixing scaffold, or the like is necessary. According to the present invention, without requiring such means, cells which may have pluripotency included in the synthetic tissue or complex can be stably accepted by the implantation portion without an additional fixing means.

(Preparation of synthetic tissue using an ECM synthesis promoting agent)

In another aspect, the present invention provides a method for producing a synthetic tissue. The method for

producing a synthetic tissue comprises the steps of: A) providing a cell; B) placing the cell in a container containing a cell culture medium including an ECM synthesis promoting agent, wherein the container has a base with an area sufficient to accommodate a desired size of the synthetic tissue; and C) culturing the cell in the container for a period of time sufficient to form the synthetic tissue having the desired size.

The above-described cell may be any cell. A method for providing a cell is well known in the art. For example, a tissue is extracted and cells are isolated from the tissue. Alternatively, cells are isolated from body fluid containing blood cells or the like. Alternatively, a cell line is prepared in an artificial culture. The present invention is not limited to this. Cells used herein may be any stem cells or differentiated cells, particularly including myoblasts, mesenchymal stem cells, adipocytes, synovial cells, bone marrow cells, and the like. Examples of mesenchymal stem cells used herein include adipose tissue-derived stem cells, bone marrow-derived stem cells, and the like.

The method for producing a synthetic tissue of the present invention employs a cell culture medium containing an ECM synthesis promoting agent. Examples of such an ECM synthesis promoting agent include, but are not limited to, ascorbic acid or a derivative thereof, ascorbic acid 1-phosphate, ascorbic acid 2-phosphate, L-ascorbic acid, and the like.

The cell culture medium used in the present invention may be any medium which allows a cell of interest to grow.

Examples of such a medium include, but are not limited to, DMEM, MEM, F12, DME, RPMI1640, MCDB104, 199, MCDB153, L15, SkBM, Basal medium, and the like which are supplemented with glucose, FCS (fetal calf serum), antibiotics (penicillin, streptomycin, etc.) as appropriate.

The container used in the present invention may be any container typically used in the art which has a base with an area sufficient to accommodate a desired size of the synthetic tissue. Examples of such a container include, but are not limited to, petri dishes, flasks, mold containers, and the like, and preferably containers having a large area of the base (e.g., at least 1 cm^2). The material of the container may be any material and include, but are not limited to, glass, plastic (e.g., polystyrene, polycarbonate, etc.), silicone, and the like.

In a preferable embodiment, the method for producing a synthetic tissue according to the present invention further comprises detaching a produced synthetic tissue. As used herein, the term "detach" indicates that after a synthetic tissue of the present invention is formed in a container, the synthetic tissue is removed from the container. The detachment can be achieved by, for example, physical means (e.g., pipetting of medium, etc.), chemical means (addition of a substance), or the like. In the present invention, a synthetic tissue can be detached by providing a stimulus around the synthetic tissue by physical means or chemical means, but not by aggressive means (e.g., treatement with a protein degrading enzyme, etc.) to the synthetic tissue. Thus, the present invention provides ease of handling, which cannot be conventionally achieved, and the resulting synthetic tissue is substantially intact, resulting in a

high-performance implant.

In a preferable embodiment, the present invention further comprises detaching cells which construct a synthetic tissue. In a more preferable embodiment, the detaching step includes applying a stimulus for contracting a synthetic tissue, including a physical stimulus (e.g., pipetting, etc.). Such a physical stimulus is not directly applied to the produced synthetic tissue. This is a preferable feature of the present invention. Since a physical stimulus is not directly applied to a synthetic tissue, it is possible to suppress damage to the synthetic tissue. Alternatively, the detaching step includes chemical means, such as adding an actin regulatory agent. Such an actin regulatory agent includes a chemical substance selected from the group consisting of actin depolymerizing agents and actin polymerizing agents. Examples of actin depolymerizing agents include, but are not limited to, ADF(actin depolymerizing factor), destrin, depactin, actophorin, cytochalasin, NGF (nerve growth factor), and the like. Examples of actin polymerizing agents include, but are not limited to, LPA (lysophosphatidic acid), insulin, PDGFm, chemokine, TGF b, and the like.

Though not wishing to be bound by any theory, these actin regulatory agents may cause actomyocin-based cytoskeleton to contract or extend, thereby regulating contraction and extension of a cell itself. As a result, a synthetic tissue itself may be promoted to or inhibited from being detached from the base of a container.

In another embodiment, the synthetic tissue and complex of the present invention are characterized in that

they are produced from cells which are cultured in monolayer culture. Despite monolayer culture, synthetic tissues having various thicknesses can be constructed. This is an unexpected effect. Conventionally, for example, a thick tissue cannot be constructed without using a multilayer structure when a temperature responsive sheet or the like is used. The present invention is the first to achieve a method for constructing a three-dimetional structure, which does not require a scaffold and can construct the contractile organization including ten or more layers. A typical cell implantation method which does not employ a scaffold is a cell sheet engineering technique utilizing a temperature sensitive culture dish disclosed by Kushida A., Yamato M., Konno C., Kikuchi A., Sakurai Y., Okano T., J. Biomed. Mater. Res., 45:355-362, 1999. The technique has won international recognition as an original technique. However, this cell sheet technique has a problem in that a single sheet is weak in many cases, and requires modification such as layering sheets for obtaining the strength resistant to an surgical operation such as implantation.

A cell/matrix complex developed by the present invention does not require a temperature sensitive culture dish unlike the cell sheet technique. The cell/matrix complex is easy to form into a contractile three-dimensional tissue. There is no technique in the world other than the present invention, which can produce a contractile three-dimensional complex having 10 or more layers without using so-called feeder cells, such as rodent stroma cells, after approximately three weeks. By adjusting conditions for matrix production of the synovial cell, it is possible to produce a complex having a strength which allows surgical manipulation, such as holding or transferring the complex,

without a special instrument. Therefore, the present invention is an original, epoch-making technique in the world for reliably and safely perform cell implantation.

In a preferable embodiment, the ECM synthesis promoting agent used in the method for producing a synthetic tissue of the present invention includes ascorbic acid 2-phosphate (Hata R., Senoo H., J. Cell Physiol., 1989, 138(1):8-16). In the present invention, by adding a certain amount or more of ascorbic acid 2-phosphate, it is possible to promote production of an extracellular matrix, so that the resultant synthetic tissue or complex is made strong to become easy to be detached. Thereafter, self contraction is elicited by applying a stimulus for detachment. Hata et al. do not report that, after adding such an ascorbic acid and culturing, a tissue becomes strong and obtains a property to be easy to be detached. Though not wishing to be bound by any theory, a significant difference is that Hata et al. used a significantly different cell density. Hata et al. does not suggest an effect of making a tissue rigid. Such an effect that the tissue is made rigid, an effect of contraction, and an effect that the tissue becomes easy to be detached are first found in the present invention. The synthetic tissue according to the present invention is recognized to be totally different from the synthetic tissue which has been fabricated conventionally at least on the point that it is produced through the process of making rigid, contraction, and detachment.

Conraction when the culture is detached and promotion in constructing a three-dimensional structure, a contractile three-dimensional tissue, or the like are suprising effects. Such effects have not been reported conventionally.

In a preferable embodiment, ascorbic acid 2-phosphate used in the present invention typically has a concentration of at least 0.01 mM, preferably at least 0.05 mM, more preferably at least 0.1 mM, even more preferably at least 0.2 mM, still more preferably at least 0.5 mM, and still even more preferably 1.0 mM. Herein, any concentration of 0.1 mM or higher may be employed. However, there may be an aspect in which a concentration of 10 mM or lower is desired.

In a certain preferable embodiment, the ECM synthesis promoting agent of the present invention includes ascorbic acid 2-phosphate or a salt thereof, and L-ascorbic acid or a salt thereof.

In a preferable embodiment, after the culturing step, the synthetic tissue production method of the present invention further comprises, detaching the synthetic tissue and allowing the synthetic tissue to perform self contraction. The detachment can be accelerated by applying a physical stimulus (e.g., application of shear stress, pipetting, deformation of the container, etc.). Self-contraction naturally takes place when a stimulus is applied after the detachment. When a chemical stimulus is applied, self-contraction and detachment occurs simultaneously. By self-contraction, biological integration is accelerated particularly in the third dimension (the direction perpendicular to the two-dimensional directions in the case of tissue on a sheet). Therefore, a synthetic tissue of the present invention may have a three-dimensional structure.

In a synthetic tissue production method of the present

invention, the sufficient time preferably means at least 3 days, though it varies depending on the application of a synthetic tissue of interest. An exemplary period of time is 3 to 7 days.

In another embodiment, the synthetic tissue production method of the present invention may further comprise causing a synthetic tissue to differentitate. By differentiation, the synthetic tissue can have a form closer to that of a desired tissue. An example of such differentiation is, but is not limited to, chondrogenesis and osteogenesis. In a preferable embodiment, osteogenesis may be performed in medium containing dexamethasone, β-glycerophosphate, and ascorbic acid 2-phosphate. More preferably, bone morphogenetic proteins (BMPs) are added. This is because such BMP-2, BMP-4, and BMP-7 proteins promote osteogenesis.

In another embodiment, a method of producing the synthetic tissue of the present invention is a process of differentiating a synthetic tissue. A form of differentiation includes performing a differentiation of cartilage. In the preferable embodiment, chondrogenesis is performed in a medium including pyruvic acid, dexamethasone, ascorbic acid 2-phosphate, insulin, transferrin, and selenious acid. More preferably, bone morphogenetic proteins (such as BMP-2, BMP-4, BMP-7), transforming growth factors (such as TGF-β1, TGF-β3) are added. This is because such BMPs promote chondrogenesis.

An important point in the present invention is that it is possible to fabricate a tissue having a pluripotency into various differentiated cells such as bone, cartilage,

and the like. Conventionally, differentiation into a cartilage tissue is difficult in other synthetic tissues which are scaffold-free. If a certain size is required, conventionally, it was necessary to coculture with a scaffold, construct a three-dimensional structure, and add a chondrogenesis medium. Conventionally, scaffold-free differentiation into cartilage was dificult. The present invention is the first to enable differentiation into cartilage in a synthetic tissue. This is not an effect which has not been obtained conventionally, and is a characteristic effect of the present invention. In a treatment which aims to regenerate a tissue, a method for performing a treatment efficiently and safely by using a tissue of sufficient size without a scaffold was difficult. The present invention achieves a significant effect on this point. Particularly, the present invention is significant on the point that it becomes possible to easily manipulate differentiated cells such as cartilage, which has been impossible conventionally. Conventionally, for example, cells can be collected to a pellete shape and the aggregation of cells can be differentiated to obtain a tissue of about 2 mm^3. For obtaining a tissue larger than this size, it was necessary to use a scaffold.

The differentiation step in synthetic tissue production of the present invention may be performed before or after providing cells.

In the present invention, primary culture cells can be used. The present invention is not limited to this. Subcultured cells (e.g., three or more passages) can also be used. Preferably, when subculture cells are used, the cells are preferably of four passages or more, more preferably

of 5 passages or more, and even more preferably of 6 passages or more. The upper limit of cell density is increased with an increase in the number of passages within a certain range. This is because a denser synthetic tissue can be produced. The present invention is not limited to this. It seems that a certain range of passages (e.g., 3 to 8 passages) are preferable.

In the present invention, the cells are preferably provided at a cell density of $5.0\times10^4/cm^2$ or more. The present invention is not limited to this. This is because a higher cell density can provide a synthetic tissue having a greater strength. It will be understood that the lower limit of the cell density may be lower than the above-described density. It will also be understood that those skilled in the art can define the lower limit based on the present specification.

In one embodiment of the present invention, for example, a myoblast, a synovial cell, an adipocyte, and a mesenchymal stem sell (e.g., derived from adipose tissue or bone marrow) can be used. The present invention is not limited to this. These cells can be applied to, for example, a heart, a bone, a cartilage, a tendon, a ligament, a joint, a meniscus, and the like.

(Synthetic tissue and complex)

In another aspect, the present invention provides a functional synthetic tissue or complex. The functional synthetic tissue of the present invention is herein an implantable synthetic tissue. Attempts have been heretofore made to produce synthetic tissues by cell culture. However, there were no synthetic tissues suitable for implantation in terms of size, strength, physical injuries

when it is detached from a culture container, or the like. The present invention provides a tissue culture method in which cells are cultured in the presence of an ECM synthesis promoting agent as described above, so that there is no problem in terms of size, strength, and the like and there is no difficulty in detaching tissues. An implantable synthetic tissue is provided only after such a tissue culture method is achieved.

Another aspect of the present invention provides cells, and a complex including factors derived from the cells. Herein, it is recognized that, preferably, the complex substantially comprises cells, and the factors derived from the cells. Herein, the complex of the present invention is provided for reinforcing, repairing, or regenerating a part of an organism.

As used herein, the term "complex" means that cells and other components are integrated into a complex by some kind of interactivity. Therefore, the complex of the present invention often has an appearance like a synthetic tissue, and it is recognized that the meaning of the term "complex" overlaps with what is referred to by a synthetic tissue.

The present invention provides a scaffold-free synthetic tissue or complex. A therapeutic method and a therapeutic agent for providing an excellent condition after implantation can be obtained by providing such a scaffold-free synthetic tissue.

The scaffold-free synthetic tissue of the present invention solves a long outstanding problem with biological formulations, which is attributed to contamination of the

scaffold itself. Despite the absence of a scaffold, the therapeutic effect is comparable with, or more satisfactory than, conventional techniques.

In addition, when a scaffold is used, the alignment of implanted cells in the scaffold, the cell-to-cell adhesion, the in vivo alteration of the scaffold itself (eliciting inflammation), the acceptance of the scaffold by the recipient tissue, and the like become problematic. These problems can be solved by the present invention.

The synthetic tissue and the complex of the present invention are also self-organized, and have biological integration inside thereof. Also in this point, the present invention is distinguished from conventional cell therapies.

The synthetic tissue and the complex of the present invention are easily used to form a three-dimensional structure, and is thus easy to be designed into a desired form. The versatility of the synthetic tissue and the complex of the present invention should be noted.

The synthetic tissue and the complex of the present invention have biological integration with recipient tissues, such as surrounding tissues, cells, and the like. Therefore, the post-operational acceptance is satisfactory, and cells are reliably supplied to a local site, for example. An effect of the present invention is that the satisfactory biological integration capability allows the formation of a tissue complex with another synthetic tissue or the like, resulting in a complicated therapy.

Another effect of the present invention is that

differentiation can be induced after the synthetic tissue or the complex is provided. Alternatively, differentiation is induced before providing a synthetic tissue and/or a complex, and thereafter, the synthetic tissue and/or the complex are formed.

Another effect of the present invention is that the cell implantation of the present invention provides a satisfactory replacement ability and a comprehensive supply of cells for covering an implanted site, compared to conventional cell-only implantation and sheet implantation.

The present invention provides an implantable synthetic tissue. The above-described features and effects of the present invention become it possible to treat a site which cannot be considered as an implantation site for conventional synthetic products. The present invention makes it possible to provide a synthetic tissue or a three-dimensional structure using not only a heart muscle but also cells derived from other parts. The synthetic tissue of the present invention has biological integration and actually works in implantation therpies. The synthetic tissue is first provided by the present invention, but is not provided by conventional techniques.

In addition, the present invention provides medical treatment which provides a therapeutic effect by filling, replacing, and/or covering an affected portion.

In addition, when the synthetic tissue of the present invention is used in combination with another synthetic tissue (e.g., an artificial bone made of hydroxyapatite, a microfibrous collagen medical device, etc.), the synthetic

tissue of the present invention is biologically integrated with the other synthetic tissue, so that the acceptance of the synthetic tissue can be improved to an extent which is not conventionally expected.

An extracellular matrix or a cell adhesion molecule, such as fibronectin, vitronectin, or the like, is distributed throughout the synthetic tissue of the present invention. In the cell sheet engineering, a cell adhesion molecule is localized on a surface of culture cells which is attached to a culture dish. In the sheet of the cell sheet engineering, cells are major components of the sheet. The sheet is nearly a mass of cells, on the bottom surface of which an adhesion molecule (glue) is added. The synthetic tissue of the present invention is a real "tissue" such that an extracellular matrix wraps cells. Thus, the present invention is significantly distinguished from conventional techniques.

A cell implanting method without a scaffold has been reported by Kushida A., Yamato M., Konno C., Kikuchi A., Sakurai Y., Okano T., J. Biomed. Mater. Res., 45:355-362, 1999, in which a cell sheet is produced using a temperature sensitive culture dish. Such a cell sheet engineering technique is internationally appraised due to its originality. However, a single sheet obtained by this technique is fragile. In order to obtain the strength that can withstand surgical manipulation, such as implantation, a plurality of sheets need to be assembled, for example. Such a problem is solved by the present invention.

A cell/matrix complex developed by the present invention does not require a temperature sensitive culture dish unlike the cell sheet technique. The cell/matrix

complex is easily formed into a contractile three-dimensional tissue. There is no technique in the world other than the present invention, which can produce a contractile three-dimensional complex having 10 or more layers without using so-called feeder cells, such as rodent stroma cells, after approximately three weeks. By adjusting conditions for matrix production of the synovial cell, it is possible to produce a complex having a strength which allows surgical manipulation, such as holding or transferring the complex, without a special instrument. Therefore, the present invention is an original, epoch-making technique in the world for reliably and safely performing cell implantation.

In a preferable embodiment, the synthetic tissue of the present invention has a biological integration capability to the surroundings. As used herein the term "surroundings" typically means surroundings to be implanted, and examples thereof include tissues, cells and the like. The biological integration capability with surrounding tissues, cells, and the like can be confirmed by, for example, photomicrograph, physical test, staining of a biological marker, or the like. Convetional synthetic tissues have a low affinity for adjacent tissues in which they are implanted. It was not even assumed that convetional synthetic tissues have the biological integration capability. Conventional synthetic tissues depend on a regeneration capability of an organism, and serves as a temporary solution until autologous cells gather and regenerate. These conventional synthetic tissues are not intended to for a permanent use. Therefore, the synthetic tissue of the present invention should be contemplated as an implantation treatment in the true sense. The biological integration capability referred to by in the present invention preferably includes an adhesion capability

to surrounding cells. Such an adhesion capability can be measured by an *in vitro* culturing assay (see Figure **23**) with a tissue section (e.g., a cartilage section).

As used herein, the term "disease" to be treated by the present invention refers to any disease accompanying degeneration, necrosis, injury or the like, and examples thereof including, osteoarthritis, osteochondral injury, intractable fracture, osteonecrosis, cartilage injury, meniscus injury, ligament injury, tendon injury, cartilage degeneration, meniscus degeneration, intervertebral disk denaturation, ligament degeneration, or tendon degeneration, or any heart diseases having an injured tissue. Examples of such heart diseases include heart failure, intractable heart failure, myocardial infarct, cardiomyopathy, dilated cardiomyopathy, hypertrophic cardiomyopathy, dilated phase hypertrophic cardiomyopathy, and the like. The combined therapy of the present invention may be applied to a regeneration of an injury in an organ other than a heart, as long as regeneration of a tissue injury is the goal. In a specific embodiment, a disease to be treated by the method of the present invention is intractable heart failure.

As used herein, the term "prophylaxis" or "prevention" in relation to a certain disease or disorder refers to a treatment which keeps such a condition from happening before the condition is caused, or causes the condition to occur at a reduced level or to be delayed.

As used herein, the term "therapy" in relation to a certain disease or disorder means that when such a condition occurs, such a disease or disorder is prevented from deteriorating, preferably is retained as it is, more preferably is diminished, and even more preferably

extinguished. As used herein, the term "radical therapy" refers to a therapy which eradicates the root or cause of a pathological process. Therefore, when a radical therapy is made for a disease, there in principle is no recurrence of the disease.

As used herein, the term "prognosis" is also referred to as "prognostic treatment". The term "prognosis" in relation to a certain disease or disorder refers to a diagnosis or treatment of such a condition after a therapy.

In a preferable embodiment, the synthetic tissue or complex of the present invention has a three-dimensional, biological integration. As described in other portions of the specification, examples of biological integration include, but are not limited to, physical integration or connection via extracellular matrices, electrical integration, and the like. Particularly, in a preferable embodiment including the cells, it is important that extracellular matrix in a tissue is biologically organized. Such a synthetic tissue which is biologically organized has not been provided. Thus, the synthetic tissue of this embodiment according to the present invention is new also in view of the structure. Further, the preferable embodiment having a biological integration capability with the surroundings provides a synthetic tissue which has not exist conventionally on the point that the synthetic tissue can form a part of an organism after implantation. The present invention can provide an synthetic tissue which does not include any cell, even a cell which has been frozen once and died. The tissue is still unique on the point that it has an affinity with the surrounding even in such a case.

In one embodiment, the synthetic tissue of the present invention is different from conventional synthetic tissues in that the former comprises a cell. Particularly, a high density that the density of $5\times10^6/cm^2$ at maximum can be included is important. The present invention is important on tha point that it is suitable for implanting cells rather than implanting the tissue.

Preferably, a synthetic tissue of the present invention substantially comprise cells or a material derived from the cells. Since the synthetic tissue is composed substantially of only cells and a cell-derived material (e.g., extracellular matrix, etc.), the synthetic tissue can have an increased level of biocompatibility and affinity. As used herein, the terms "substantially comprise ...", "substantially made of ...", and "substantially contain ..." mean that cells and substanced derived from the cells are included, and also any other substance may be included as long as it does not cause any harmful effect (herein, mainly, bad effect on implantation), and should understood as such herein. Such substances which do not cause any harmful effect are known to those skilled in the art or can be confirmed by conducting an easy test. Typically, such substances are, but not limited to, any additives permitted by the Health, Labor and Welfare Ministry, Food and Drug Administration (FDA) or the like, ingredients involved in cell culture, and the like. The cell-derived material representatively includes extracellular matrices. Particularly, the synthetic tissue or complex of the present invention preferably comprises a cell and an extracellular matrix at an appropriate ratio thereof. Such an appropriate ratio of a cell and an extracellular matrix is from about 1:3 to about

20:1. The strength of the tissue is adjusted by the ratio between a cell and an extracellular matrix. The ratio between a cell and an extracellular matrix is adjusted for use in accordance with application of cell implantation and physical environment at the implantation site. Preferable ratio varies depending on the treatment to be aimed. Such a variation is apprarent to those skilled in the art and can be estimated by investigating the ratio of a cell in an organ which is a target and an extracellular matrix.

Preferablly, a synthetic tissue substantially comprising cells and an extracellular matrix derived from the cells has not been known. Therefore, the present invention provides a totally new synthetic tissue.

Preferably, an extracellular matrix which forms the present invention includes, collagen I, collagen III, vitronectin, fibronectin, and the like. It is preferable that a variety of extracellular matrix includes all the listed ingredients, and that they are integrated and mixed. Alternatively, it is preferable that extracellular matrix is dispersed across the entire body. Such a distribution has a significant effect on the point that compatibility and affinity with the environment can be improved when implanted. The present invention is known to be characterized in that adhesion to intercellular matrix which promotes cell adhesion to a matrix, cell extension, and cell chemotaxis is also promoted by including collagen (Types I, III), vitronectin, fibronectin, and the like. However, a synthetic tissue which includes collagen (Types I, III), vitronectin, fibronectin, and the like has not been provided. It is not intended to be constrained by the theory, but, collagen (Types I, III), vitronectin, fibronectin, and the

like are contemplated to have a function in exercising the biological integration capability with the surrounding. Therefore, in the preferable embodiment, it is advantageous that vitronectin are positioned to be dispersed on a surface of the synthetic tissue or complex of the present invention. It is considered that adhesion, affinity, and stability after implantation are significantly different.

It is preferable that the fibronectin is also positioned in the synthetic tissue or complex of the present invention. It is known that fibronectin has a function in cell adhesion, control of a shape of a cell, and adjustment in cell migration. A synthetic tissue in which fibronectin is expresse has not been provided. It is not intended to be contrained by the theory, fibronection is also contemplated to have a function in exercising the biological integration capability with the surrounding. Therefore, in the preferable embodiment, it is advantageous that fibronectin are also positioned to be dispersed on a surface of the synthetic tissue or complex of the present invention. It is considered that adhesion, affinity, and stability after implantation are significantly different.

In the preferred embodiment, it is understood that to position extracellular matrix used in the present invention on the synthetic tissue or complex can be readily achieved by the synthetic tissue production method of the present invention. It is also understood that the production method is not limited to this.

In more preferable embodiment, it is advantageous to position the extracellular matrix used in the present invention to be disperesed. Positioning extracellular

matrix into such a dispered state was impossible in convetional synthetic tissues. It is understood the present invention is the fist to provide such a tissue.

In the preferred embodiment, regarding extracellular matrix positioned to be dispersed on the synthetic tissue or complex, when distribution densities in any two section of 1 cm^2 are compared, the ratio is preferably within the range of about 1:3 to 3:1. Measurement of distribution densities can be performed by any method known in the field of the art, for example, immune stainintg or the like.

In the preferred embodiment, regarding extracellular matrix used in the present invention, when distribution densities in any two section of 1 cm^2 are compared, the ratio is preferably within the range of about 1:2 to 2:1, and further preferably, about 1.5:1 to 1.5:1. It is advantageous that extracellular matrix is uniformly disperesed. Preferably, extracelluar matrix is dispersed substantially uniform, but it is not limited to this.

In one embodiment, extracellular matrix positioned in the present ivnetion may include collagen I, collagen III, vitronectin, fibronectin or the like.

In an alternative embodiment, the synthetic tissue or complex of the present invention may employ heterologous cells, allogenic cells, isogenic cells or autologous cells. In the present invention, it is found that even allogenic cells, particularly, mesenchymal cells are used, no adverse reactions , such as immune rejection reactions, is generated. Thus, the present invention ends to the development of the

treatment of *ex vivo*, and also a therapy which produces a synthetic tissue using cells of others and utilize the tissue without using an immuno rejection suppressor or the like.

In one preferred embodiment, the cells included in the synthetic tissue or complex of the present invention may be stem cells, differentiation cells, or they may include both. In the preferred embodiment, the cells included the three directional sturucture are mesenchymal cells. It is not intended to restrained to the theory, the mesenchymal cells are preferably used because the mesenchymal cells are highly compatible with various organs such as heart, and may have capability to differentiate into various organs such as a heart.

Such mesenchymal cells may be mesenchymal stem cells, or may be mesenchymal differentiation cells.

Examples of the mesenchymal cells used in the present invention include, but not limited to, bone marrow cells, adipocyte, synovial cell, myoblast, skeletal muscle cells, and the like. Examples of mesenchymal cells as used herein include stem cells derived from an adipose tissue, stem cells derived from a bone marrow, and the like.

In the preferred embodiment, it is advantageous that the cells used in the present invention are cells derived from the subject to which the synthetic tissue or complex is applied. In such a case, cells as used herein also referred to as autologous cells. By using autologous cells, immune rejection reactions can be prevented or reduced.

Alternatively, in another embodiment, the cells as

used herein may not be cells derived from a subject to which the synthetic tissue or complex is applied. In such a case, it is preferable that measures are taken to prevent immune rejection reactions.

The synthetic tissue or complex of the present invention may be provided as a drug. Alternatively, the synthetic tissue or complex may be prepared by a physician for therapy, or, a physician may first prepare the cells, and then the third party may culture the cells and prepare as a third-dimension structure for use in a surgery. In such a case, culturing cells is not necessarily performed by a physician, but can be performed by those skilled in the art of cell culture. Those skilled in the art can determine culturing conditions in accordance with a variety of the cells and an implantation site to be targeted after reading the disclosure herein.

In another embodiment, the synthetic tissue or complex of the present invention is preferably isolated. In this case, the term "isolate" means that the synthetic tissue is detached from a scaffold, a support, and a culture medium used in culture. If a synthetic tissue of the present invention is substantially free of materials, such as a scaffold and the like, it is possible to suppress adverse reactions after implantation, such as immune rejection reactions, inflammation reactions, and the like.

The base area of the synthetic tissue according to the present invention may be, for example, 1 cm^2 to 20 cm^2. However, the area is not limited to this range and may be smaller than 1cm^2, or greater than 20cm^2. It is understood

that the essential feature of the present invention is that a tissue of any size (area, volume) can be produced, and it is not limited in the size.

In a preferable embodiment, the synthetic tissue of the present invention is thick. The term "thick" in relation to a synthetic tissue typically means that the synthetic tissue has a thickness which provides a strength sufficient to cover a site to which the synthetic tissue is implanted. Such a thickness is, for example, at least about 50 μm, more preferably at least about 100 μm, at least about 200 μm, at least about 300 μm, even more preferably at least about 400 μm, still more preferably at least about 500 μm, and still even more preferably about 1 mm. It is recognized that, in some cases, a tissue having a thickness of 3 mm or greater and a tissue having a thickness of 5 mm or greater can be produced. Alternatively, such a thickness may be, 1 mm or less. It is understood that an essential feature of the present invention is that a tissue or a complex having any thickness can produced, and the tissue or complex is not limited in the size.

The present invention provides a scaffold-free synthetic tissue or complex. By providing such a scaffold-free synthetic tissue, a therapeutic method and a therapeutic agent for providing an excellent condition after implantation can be obtained.

The scaffold-free synthetic tissue of the present invention solves a long outstanding problem with biological formulations, which is attributed to contamination of the scaffold itself. Despite the absence of a scaffold, the therapeutic effect is comparable with or more satisfactory

than conventional techniques.

In addition, when a scaffold is used, the alignment of implanted cells in the scaffold, the cell-to-cell adhesion, the in vivo alteration of the scaffold itself (eliciting inflammation), the acceptance of the scaffold to recipient tissue, and the like become problematic. These problems can be solved by the present invention.

The synthetic tissue and the complex of the present invention are also self-organized, and have biological integration inside thereof. Also in this point, the present invention is distinguished from conventional cell therapies.

The synthetic tissue and the complex of the present invention are easy to form a three-dimensional structure, and is thus easy to be designed into a desired form. The versatility of the synthetic tissue and the complex of the present invention should be noted.

The synthetic tissue and the complex of the present invention have biological integration with recipient tissues, such as surrounding tissues, cells, and the like. Therefore, the post-operational acceptance is satisfactory, and cells are reliably supplied to a local site, for example. An effect of the present invention is that the satisfactory biological integration capability allows the formation of a tissue complex with another synthetic tissue or the like, resulting in a more complex therapy.

Another effect of the present invention is that differentiation can be induced after the synthetic tissue or the complex is provided. Alternatively, differentiation

is induced before providing a synthetic tissue and/or a complex, and thereafter, the synthetic tissue and/or the complex are formed.

Another effect of the present invention is that the cell implantation of the present invention provides a satisfactory replacement and a comprehensive supply of cells for covering an implanted site, compared to conventional cell-only implantation and sheet implantation.

The present invention provides an implantable synthetic tissue having biological integration capability. The above-described features and effects of the present invention become it possible to treat a site which cannot be considered as an implantation site for conventional synthetic products. The present invention makes it possible to provide a synthetic tissue or a three-dimensional structure. The synthetic tissue of the present invention has biological integration and actually works in implantation therapies. The synthetic tissue is first provided by the present invention, but is not provided by conventional techniques.

In addition, the present invention provides medical treatment which provides a therapeutic effect by filling, replacing, and/or covering an affected portion.

In addition, when the synthetic tissue of the present invention is used in combination with another synthetic tissue (e.g., an artificial bone made of hydroxyapatite, a microfibrous collagen medical device, etc.), the synthetic tissue of the present invention is biologically integrated with the other synthetic tissue, so that the acceptance of

the synthetic tissue can be improved to an extent which is not conventionally expected.

An extracellular matrix or a cell adhesion molecule, such as fibronectin, vitronectin, or the like, is distributed throughout the synthetic tissue of the present invention. In cell sheet engineering, a cell adhesion molecule is localized on a surface of culture cells which is attached to a culture dish. In the sheet of the cell sheet engineering, the cells are major components of the sheet. The sheet is nearly a mass of cells, on the bottom surface of which an adhesion molecule (glue) is added. On the other hadn, the synthetic tissue of the present invention is a real "tissue" such that an extracellular matrix covers cells. Thus, the present invention is significantly distinguished from conventional techniques.

A cell implanting method without a scaffold has been reported by Kushida A., Yamato M., Konno C., Kikuchi A., Sakurai Y., Okano T., J. Biomed. Mater. Res., 45:355-362, 1999, in which a cell sheet is produced using a temperature sensitive culture dish. Such a cell sheet engineering technique is internationally appraised due to its originality. However, a single sheet obtained by this technique is fragile. In order to obtain the strength that can withstand surgical manipulation, such as implantation, a plurality of sheets need to be assembled, for example. Such a problem is solved by the present invention.

A cell/matrix complex developed by the present invention does not require a temperature sensitive culture dish unlike the cell sheet technique. The cell/matrix complex is easy to form into a contractile three-dimensional

tissue. There is no technique in the world other than the present invention, which can produce a contractile three-dimensional complex having 10 or more layers without using so-called feeder cells, such as rodent stroma cells, at about three weeks. By adjusting conditions for matrix production of the cell, it is possible to produce a complex having a strength which allows surgical manipulation, such as holding or transferring the complex, without a special instrument. Therefore, the present invention is an original, epoch-making technique in the world for reliably and safely performing cell implantation.

In another embodiment, the synthetic tissue or complex of the present invention is flexible. Due to the flexibility, the synthetic tissue is particularly suitable for reinforcement of motile organs. Examples of motile organs include, but are not limited to, hearts, blood vessels, muscles, and the like.

In another embodiment, the synthetic tissue or complex of the present invention has dilation/contraction ability. Due to the dilation/contraction ability, the synthetic tissue is suitable for organs which expand and contract, including, for example, hearts, muscles, and the like. The dilation/contraction ability cannot be achieved by cell sheet or the like prepared by conventional methods. Preferably, a synthetic tissue of the present invention has a sufficient strength to withstand the pulsation motion of a heart. The strength sufficient to withstand pulsation motion is, but is not limited to, at least about 50% of the strength of naturally-occurring myocardium, preferably at least about 75%, and more preferably at least about 100%.

In a preferable embodiment, the synthetic tissue or complex of the present invention has biological integration in all three dimensions. There are some synthetic tissues prepared by conventional methods, which have biological integration in two dimensions to some degree. However, no tissue having biological integration in all three dimensions can be prepared by conventional methods. Therefore, since the synthetic tissue of the present invention has biological integration in all three dimensions, the synthetic tissue is substantially implantable in any application.

Examples of biological integration which is an indicator of a synthetic tissue or complex of the present invention, include, but are not limited to, interconnection of extracellular matrices, electrical integration, the presence of intracellular signal transduction, and the like. The interaction of extracellular matrices can be observed with a microscope by staining intracellular adhesion as appropriate. Electrical integration can be observed by measuring electric potential.

In a preferable embodiment, the synthetic tissue of the present invention has a sufficient tissue strength for clinical applications. The sufficient tissue strength for clinical applications varies depending on a site to which the synthetic tissue is applied. Such a strength can be determined by those skilled in the art with reference to the disclosure of the specification and techniques well known in the art. The tensile strength of the synthetic tissue of the present invention may be low. The tensile strength becomes higher when the matrix concentration is increased, and becomes lower when the cell ratio is increased. The present invention is characterized in that the strength can

be adjusted as necessary. The present invention is also characterized in that the strength can approximate to be high or low relative to that of a tissue to be implanted. Therefore, it is recognized that the goal can be set to comply with any site.

In another embodiment, it is preferable that a strength of the synthetic tissue or complex is sufficient for having a self-supporting ability. Conventional synthetic tissues do not have a self-supporting ability after production. Therefore, when conventional synthetic tissues are transferred, at least a part of them are injured. However, when the technique of the present invention is used, the synthetic tissue having the self-supporting ability is provided. This means that the present invention provides the synthetic tissue which cannot be provided by conventional techniques. Preferable self-supporting ability is such that, when a tissue is picked up with a tweezers having tips of 0.5 to 3 mm (preferably, tips of 1 to 2 mm, and more preferably, tips of 1 mm), the tissue is not substantially destroyed. Herein, whether the tissue is not substantially destroyed can be confirmed with eyes, but can be confirmed by performing, for example, a water leakage test after the tissue is picked up in the above-described conditions and confirming that water does not leak. Alternatively, the self-supporting ability as described above can also be confirmed by not being destroyed when picked up by fingers, instead of tweezers.

In a particular embodiment of the present invention, the above-described clinical application is intended to a bone, a joint, a cartilage, a meniscus, a tendon, a ligament, a kidney, a liver, a synovial membrane, a heart, and the

like. The origin of cells contained in the synthetic tissue of the present invention is not affected by clinical applications.

Also, when a synthetic tissue of the present invention is applied to a cartilage, the attachment ability of the synthetic tissue can be tested by determining whether or not the synthetic tissue remains attached without an additional fixation procedure when the synthetic tissue is implanted into an injured portion of the intra-articular tissue (e.g., 2, 3 minutes after).

In another aspect, the present invention provides a cell culture composition for producing synthetic tissue from a cell. The cell culture composition contains an ingredient (e.g., commercially available medium, etc.) for maintaining or growing the cell, and an ECM synthesis promoting agent. The ECM synthesis promoting agent has been described in detail in the above description of the synthetic tissue production method. Therefore, the ECM synthesis promoting agent includes ascorbic acid or a derivative thereof (e.g., TGF-β1, TGF-β3, ascorbic acid 1-phosphate or a salt thereof, ascorbic acid 2-phosphate or a salt thereof, L-ascorbic acid or a salt thereof, etc.). The culture composition of the present invention contains ascorbic acid 2-phosphate or a salt thereof at a concentration of at least 0.1 mM. Alternatively, in the case of a condensed culture composition, the condensed culture composition contains ascorbic acid 2-phosphate or a salt thereof at a concentration which becomes at least 0.1 mM after preparation. Ascorbic acid 2-phosphate or a salt thereof contained in the culture composition of the present invention is present at a concentration of at least 0.1 mM. When the culture

composition of the present invention is condensed, ascorbic acid 2-phosphate or a salt thereof contained therein is present at a concentration of at least 0.1 mM after fomulation. It seems that 0.1 mM or more ascorbic acids have substantially a constant effect. Thus, 0.1 mM can be said to be sufficient. For TGF-β1 and TGF-β3, 1 ng/ml or more, representatively 10 ng/ml, may be sufficient.

Alternatively, the present invention may provide a composition for producing a synthetic tissue, comprising such an ECM synthesis promoting agent.

In another embodiment of the present invention, an ECM synthesis promoting agent used in the synthetic tissue production method of the present invention includes ascorbic acid 2-phosphate (Hata R., Senoo H., J. Cell Physiol., 1989, 138(1):8-16). In the present invention, by adding an at least predetermined amount of ascorbic acid 2-phosphate, the production of an extracellular matrix is promoted. As a result, the resultant synthetic tissue or complex is made rigid, and therefore, becomes easy to be detached. Thereafter, the tissue undergoes self-contraction in response to a stimulus of detachment. Hata et al. does not disclose that the culture in medium supplemented with ascorbic acid causes the tissue to be rigid and thus confers to the tissue a property of being easily detached. Though not wishing to be bound by any theory, a significant difference between the present invention and Hata et al. is present in cell density. Also, Hata et al. does not suggest the effect of facilitating detachment of cells from a container for culture. The present invention is the first to find the effect of tissue contraction on development of three-dimensional synthetic tissue from monolayer cultured

cells. The synthetic tissue of the present invention can be absolutely distinguished from conventional synthetic tissues, since the synthetic tissue of the present invention is produced via the procedures of tissue detachment and subsequent tissue contraction.

In a preferable embodiment, ascorbic acid 2-phosphate used in the present invention is typically present at a concentration of at least 0.01 mM, preferably at least 0.05 mM, more preferably at least 0.1 mM, even more preferably at least 0.2 mM, and still more preferably at least 0.5 mM, and still even more preferably 1.0 mM.

In one embodiment of the present invention, the cell density is, but is not particularly limited to, 5×10^4 to 5×10^6 cells per 1 cm^2. These conditions may be, for example, applied to myoblast. In this case, preferably, the ECM synthesis promoting agent may be ascorbic acids and may be provided at a concentration of at least 0.1 mM. This is because a thick synthetic tissue can be produced. In this case, if the concentration is increased, a synthetic tissue having a dense extracellular matrix is produced. If the concentration is low, the amount of an extracellular matrix is decreased but the self-supporting ability is maintained.

(Synthetic tissue for replacement and coverage)

In another aspect, the present invention provides a synthetic tissue or complex for reinforcement of a portion of an animal organism. The synthetic tissue or complex capable of such reinforcement is a technique achieved only after the synthetic tissue production method of the present invention is provided. Since the synthetic tissue or complex of the present invention has self-supporting ability, it

can be used in applications which are not conventionally provided (e.g., filling (replacement) reinforcement, whole reinforcement, no-leakage reinforcement, coverage, etc.). The present invention has a significant effect such that the filling and replacement reinforcement (i.e., cell supply) was significantly improved. The present invention also allows differentiation induction, which enlarges the range of application of the present invention.

In a specific embodiment of the present invention, the above-described reinforcement may be achieved by disposing a synthetic tissue of the present invention to cover the above-described portion. It is not possible to use a synthetic tissue provided by conventional methods to perform treatment by covering the above-described portion (i.e., replacement and/or coverage application). Thus, the synthetic tissue of the present invention can provide applications which cannot be achieved by conventional techniques.

Therefore, in the above-described specific embodiment, the synthetic tissue or complex of the present invention is resistant to dilation/contraction of the above-described portion.

In a preferable embodiment, the synthetic tissue or complex of the present invention advantageously has biological integration.

In another preferable embodiment, the biological integration includes at least one of interconnection of extracellular matrices, electrical integration, and intracellular signal transduction.

In another preferable embodiment, the synthetic tissue or complex for reinforcement of the present invention is formed by culturing a cell in the presence of an ECM synthesis promoting agent.

In another embodiment, the synthetic tissue or complex for reinforcement of the present invention comprises a cell (autologous cell) derived from an animal to be treated (e.g., a human). More preferably, a synthetic tissue for reinforcement of the present invention comprises only a cell(s) (autologous cell) derived from an animal to be treated (e.g., a human) as a cell source.

Applications for the therapy utilizing the present invention include, for example: cartilage full thickness injury, cartilage partial injury; osteochondral injury; osteonecrosis; osteoarthritis; meniscus injury; ligament injury (chronic injury, degenerative tear, biological augmentation for reconstruction surgery, etc.); rotator cuff (particularly, chronic injury, degenerative tear, etc.); delayed union; nonunion; skeletal muscle repair/regeneration; cardiac muscle repair; (augmenting the repair of necrotic tissue by ischemic-heart disease) or the like.

(Therapy using replacement and coverage)

In another aspect, the present invention provides a method for reinforcement of a portion of an animal organism. The method comprises the steps of: A) disposing a synthetic tissue or complex to replace or cover the portion; and B) holding the synthetic tissue or complex for a time sufficient to connect to the portion. Herein, to position

a portion for replacement typically means to perform debridement or curettage of an affected portion as necessary, to position the synthetic tissue or complex of the present invention on the lesion, and to allow it to stand so as to promote replacement. An objective of such replacement is to fill cells. Techniques known in the art can be combined and used. The step of disposing the synthetic tissue to cover the portion can be carried out using a technique well known in the art. The sufficient time varies depending on a combination of the portion and the synthetic tissue, and can be easily determined as appropriate by those skilled in the art depending on the combination. Examples of such a time include, but are not limited to, 1 week, 2 weeks, 1 month, 2 months, 3 months, 6 months, 1 year, and the like. In the present invention, a synthetic tissue preferably comprises substantially only cell(s) and material(s) derived from the cell. Therefore, there is no particular material which needs to be extracted after operation. The lower limit of the sufficient time is not particularly important. In this case, it can be said that the longer the time, the more preferable the synthetic tissue. If the time is sufficiently extremely long, it can be said that reinforcement is substantially completed. Therefore, the time is not particularly limited. The synthetic tissue of the present invention is also characterized in that it is easily handled, is not destroyed during an actual treatment, and facilitates a surgery due to its self-supporting ability.

In another embodiment, in a reinforcement method of the present invention, the above-described portion preferably includes bag-shaped organs (e.g., hearts, livers, kidneys, etc.). In order to reinforce such a bag-shaped tissue, it is necessary to replace or cover the organ. A

synthetic tissue resistant to applications for replacement or covering is first provided by the present invention. Therefore, the reinforcement method of the present invention is advantageous over conventional techniques.

Alternatively, the above-described portion may include a bone or cartilage. Examples of such portions include, but not limited to, meniscus, ligament, tendon, and the like. By the method of the present invention a disease, injury, or condition of a heart, bone, cartilage, ligament, tendon, or meniscus may be treated, prevented or reinforced.

Particularly, in the reinforcement method of the present invention, a synthetic tissue or complex of the present invention is resistant to dilation/contraction of the above-described portion. Examples of such dilation/contraction include, but are not limited to, the pulsation motion of a heart, the contraction of a muscle, and the like.

In another preferable embodiment, in the reinforcement method of the present invention, a synthetic tissue or complex of the present invention has biological integration (e.g., interconnection of extracellular matrices, electrical integration, intracellular signal transduction, etc.). The biological integration is preferably provided in all three dimensions.

In another preferable embodiment, the reinforcement method of the present invention further comprises culturing a cell in the presence of an ECM synthesis promoting agent to form a synthetic tissue or complex of the present invention.

An implantation/regeneration technique using the method which comprises the step of culturing a cell in the presence of an ECM synthesis promoting agent cannot be provided by conventional techniques. The method provides a therapy for diseases (e.g., , cartilage injury, intractable bone fracture, etc.), which cannot be achieved by conventional therapies.

In a preferable embodiment, in the reinforcement method of the present invention, the cell used in the synthetic tissue or complex of the present invention is derived from an animal to which the synthetic tissue is to be implanted (i.e., an autologous cell). By using an autologous cell, adverse side effects, such as immune rejection reactions or the like, can be avoided.

In another preferable embodiment, the portion is a heart.

Applications for the therapy utilizing the present invention include, for example: cartilage full thickness injury, cartilage partial injury; osteochondral injury; osteonecrosis; osteoarthritis; meniscus injury; ligament injury (chronic injury, degenerative tear, biological augmentation for reconstruction surgery, etc.); rotator cuff (particularly, chronic injury, degenerative tear, etc.); delayed union; nonunion; skeletal muscle repair/regeneration; cardiac muscle repair; (augmenting the repair of necrotic tissue by ischemic-heart disease) or the like.

For some organs, it is said that it is difficult to radically treat a specific disease, disorder, or condition thereof (e.g., refractory heart diseases). However, the

present invention provides the above-described effect, thereby making possible a treatment which cannot be achieved by conventional techniques. It has been clarified that the present invention can be applied to radical therapy. Therefore, the present invention has usefulness which cannot be achieved by conventional medicaments.

Thus, the present invention provides a method for treating a portion of an organism of an animal, comprising: A) positioning the synthetic tissue or complex so as to cover the portion; and B) retaining the synthetic tissue for a time period which is sufficient for the condition of the portion of the organism to be improved. Such an improvement in the condition can be determined can be determined in accordance with the function of the portion to be treated. For example, when a heart should be treated, an improvement in the condition can be determined by checking a cardiac function (heartbeat, bloodstream, or the like). If a bone should be treated, an improvement in the condition can be determined by observing osteogensis by using roentgen, CT scan, or the like. In the case of a bone, an improvement in the condition can be determined by measuring its strength or by evaluating bone marrow and/or a bone substance by using MRI. If a cartilage or meniscus should be treated, a surface of a joint can be observed by an arthroscopy. Further, it is possible to determine an improvement in the condition by performing a biomechanical inspection under arthroscopy. It is also possible to determine an improvement in the condition by confirming a repairing condition by using MRI. Regarding ligament, it is possible to determine by confirming whether there is laxity by a joint stability inspection. Further, an improvement of the condition can be determined by confirming a continuousness of a tissue by an MRI. In

the case of any tissue, it is possible to determine whether the condition is improved by performing a biopsy of the tissue and making a histological evaluation.

In a preferred embodiment the treatment treats, prevents, prognosis, or enhances a disease, injury, or condition of a heart, bone, cartilage, ligament, tendon, or meniscus. Preferably, the synthetic tissue or the complex has a self-supporting ability. For such a synthetic tissue, those skilled in the art can use a synthetic tissue of any form described above herein, and a variant thereof.

(Combined therapy)

In another aspect, the present invention provides a regeneration therapy which uses a cytokine, such as BMP (e.g., BMP-2, BMP-4, BMP-7, etc.), TGF-β1, TGF-β3, HGF, FGF, IGF, or the like, in combination with a synthetic tissue.

Some cytokines used in the present invention are already commercially available (e.g., BMP (Yamanouchi Pharmaceutical), bFGF2 (Kaken Pharmaceutical), TGF-β1 (for research only, HGF-101 from Toyo Boseki, etc.). However, these cytokines can be prepared by various methods and can be used in the present invention if they are purified to an extent which allows them to be used as a medicament. A certain cytokine can be obtained as follows: primary cultured cells or an established cell line capable of producing the cytokine is cultured; and the cytokine is separated from the culture supernatant or the like, followed by purification. Alternatively, a gene encoding the cytokine is incorporated into an appropriate vector by a genetic engineering technique; the vector is inserted into an appropriate host to transform the host; a recombinant

cytokine of interest can be obtained from the supernatant of the transformed host culture (e.g., Nature, 342, 440(1989); Japanese Laid-Open Publication No. 5-111383; Biochem-Biophys. Res. Commun., 163, 967 (1989), etc.). The above-described host cell is not particularly limited and can be various host cells conventionally used in genetic engineering techniques, including, for example, *Escherichia coli*, yeast, animal cells, and the like. The thus-obtained cytokine may have one or more amino acid substitutions, deletions and/or additions in the amino acid sequence as long as it has substantially the same action as that of the naturally-occurring cytokine. Examples of a method for introducing the cytokine into patients in the present invention include, but are not limited to, a Sendai virus (HVJ) liposome method with high safety and efficiency (Molecular Medicine, 30, 1440-1448(1993); Jikken Igaku (Experimental Medicine), 12, 1822-1826 (1994)), an electrical gene introduction method, a shotgun gene introduction method, a ultrasonic gene introduction method, and the like. In another preferable embodiment, the above-described cytokines can be administered in the form of proteins.

(Production method of synthetic tissue having desired thickness)

Another aspect of the present invention provides a method for producing a synthetic tissue or complex having a desired thickness. This method comprises: A) providing cells; B) positioning the cells in a container having the base area sufficient for accommodating the synthetic tissue or complex having the desired size, which contains an ECM synthesis promoting agent (e.g., ascorbic acids, TGF-β1, TGF-β3, etc.); C) culturing the cells in the container with

a cell culture medium including the ECM synthesis promoting agent for a time sufficient for forming the synthetic tissue or complex having the desired size to convert the cells into a synthetic tissue; and D) adjusting the thickness of the synthetic tissue to obtain a desired thickness by a physical stimulation or a chemical stimulation. Herein, the steps of providing the cells, positioning the cells, stimulating and converting into the tissue or complex are described with respect to the production method for the synthetic tissue or complex of the present invention in detail, and it is understood that any embodiment can be employed.

Next, examples of the physical or chemical stimulation to be used may include, but not limited to, use pipetting, use of actin interacting substance. Pipetting may be preferable because operation is easy and no harmful substance is produced. Alternatively, examples of the chemical stimulation to be used may include actin depolymerizing factors and actin polymerizing factor. Examples of such an actin depolymerizing factor may include ADF(actin depolymerizing factor), destrin, depactin, actophorin, cytochalasin, NGF(nerve growth factor) and the like. Examples of the actin polymerizing factor include LPA(lysophosphatidic acid), insulin, PDGFa, PDGFb, chemokine, and TGFb. The polymerization or depolymerization of actin can be observed by checking the activity to actin. It is possible to test any substance whether it has such an activity. It is understood that a substance which is tested as such and identified can be used for achieving the desired thickness in production of the synthetic tissue of the present invention. For example, in the present invention, the adjustment of the desired thickness can be achieved by adjusting the ratio between

the actin depolymerizing factor and actin polymerizing factor.

(Composite tissue)

Another aspect of the present invention also provides a tissue complex including an implantable synthetic tissue and another synthetic tissue. Herein, another tissue may either be a synthetic tissue included within the scope of the present invention, or a synthetic tissue out of the scope (i.e., conventional tissues). Conventional tissues (e.g., an artificial bone, microfibrous collagen medical device, etc.,) do not have a biological integrating ability or have a biological integrating ability which cannot stand the practical use. Thus, it was almost impossible to form such a tissue complex. It is understood that, according to the present invention, a cartilage can be combined to a bone for treatment. For the case of a cavity in a bone or the like, particularly, for the case of treatment of bone cartilage complex, by using a tissue complex of an artificial bone (e.g., hydroxyapatite construct such as NEO BONE, a microfibrous collagen medical device, etc.) and the synthetic tissue or complex of the present invention, it is possible to treat the bone by the artificial bone, and the cartilage on the bone by the synthetic tissue at the same time. It is understood that the synthetic tissue or complex of the present invention is combined to an artificial bone and used for treatment. Herein, the implantable synthetic tissue or complex of the present invention substantially comprises, for example, cells and substances derived from the cells, and more preferably, cells and extracellular matrix derived from the cells. The extracellular matrix as used herein is selected from the group consisting of collagen I, collagen III, vitronectin, and fibronectin.

As used herein, the term "tissue complex" refers to a tissue obtained by combining a synthetic tissue or complex of the present invention with another synthetic tissue (including a synthetic tissue or complex of the present invention). Such a tissue complex can be used for a treatment of a plurality of tissues. For example, such a tissue complex can be used for treatment of both cartilage and bone.

In the case there is a large defect of soft tissue (e.g., menisucus, etc.), the synthetic tissue of the present invention can be coupled to another synthetic tissue (microfibrous collagen medical device (e.g., CMI (Amgen, USA), Integran® (Nippon Zoki Pharmaceutical), hyaluronic acid gel, collagen gel, agarose gel, alginate gel, beads etc.) to promote biological integration between another synthetic tissue and an implantation cells.

Preferably, in the complex of the present invention, an implantable synthetic tissue and another synthetic tissue are biologically integrated. Such integration can be produced by culturing two tissues in contact. Such a biological integration is mediated by extracellular matrix.

Hereinafter, the present invention will be described by way of examples. Examples described below are provided only for illustrative purposes. Accordingly, the scope of the present invention is not limited except as by the appended claims.

(Examples)

In the examples below, animals were treated in

accordance with rules defined by Osaka University (Japan) and were cared for in the spirit of animal protection.

(Example 1: Synovial cell)

In this example, various synovial cells were used to produce a synthetic tissue as follows.

<Preparation of cells>

Synovial cells were collected from a knee joint of a pig (LWD ternary hybrid, 2-3 months old upon removal of cells), followed by treatment with collagenase. The cells were cultured and subcultured in 10% FBS-DMEM medium (FBS was obtained from HyClone, DMEM was obtained from GIBCO). It has been reported that 10th passage synovial cells still have pluripotency. Although cells of 10 or less passages were used in this example, cells of more than 10 passages may be used depending on the application. Autotransplantation was performed for humans, where a sufficient number of cells were used and the cells were cultured for a short period of time so as to reduce the risk of infection or the like.

Considering these points, cells of various passages were used. Actually, primary culture cells, first passage cells, second passage cells, third passage cells, fourth passage cells, fifth passage cells, sixth passage cells, eighth passage cells, and tenth passage cells were used in experiments. These cells were used for synthetic tissues.

<Preparation of synthetic tissue>

Synovial cells (4.0×10^6) were cultured in 2 ml of 10% FBS-DMEM medium in a 35-mm dish, a 60-mm dish, or 100-mm dish (BD Biosciences, culture dish and multiwell cell culture

plate). In this case, ascorbic acid was added. The dishes, the ascorbic acid concentrations, and the cell concentration are described below.

Dishes: BD Biosciences, cell culture dishes and multiwell cell culture plates

Ascorbic acid 2-phosphate: 0 mM, 0.1 mM, 0.5 mM, 1 mM, 2 mM, and 5 mM

The number of cells: 5×10^4 cells/cm^2, 1×10^5 cells/cm^2, 2.5×10^5 cells/cm^2, 4.0×10^5 cells/cm^2, 5×10^5 cells/cm^2, 7.5×10^5 cells/cm^2, 1×10^6 cells/cm^2, 5×10^6 cells/cm^2, and 1×10^7 cells/cm^2

Medium was exchanged two times per week until the end of a predetermined culture period. At the end of the culture period, a cell sheet was detached from the dish by pipetting circumferentially around the dish using a 100-µl pipetteman. After detachment, the cell sheet was made as flat as possible by lightly shaking the dish. Thereafter, 1 ml of medium was added to completely suspend the cell sheet. The cell sheet was allowed to stand for two hours, resulting in the contraction of the cell sheet into a three-dimensional form. Thus, a synthetic tissue was obtained (Fig. 1).

<Hematoxylin-Eosin (HE) Staining>

The acceptance or vanishment of cells in a sheet was observed by HE staining. The procedure is described as follows. A sample is optionally deparaffinized (e.g., with pure ethanol), followed by washing with water. The sample is immersed in Omni's hematoxylin for 10 min. Thereafter, the sample is washed with running water, followed by color

development with ammonia in water for 30 sec. Thereafter, the sample is washed with running water for 5 min and is stained with eosin hydrochloride solution for 2 min, followed by dehydration, clearing, and mounting.

(Various extracellular matrix staining)

1. Make 5 μm thick sections from frozen block.
2. Sections are fixed in acetone at -20°C for 5-10 mins.
   (Paraffin blocks should be deparaffinized and rehydrated).
3. Endogenous peroxide activity is blocked in 0.3% H_2O_2 in methanol for 20 mins at RT.
   (1 ml 30% H_2O_2 + 99 ml methanol)
4. Wash with PBS (3 × 5 mins).
5. Incubate with primary monoclonal antibody (a mouse or rabbit antibody against each extracellular matrix protein) in a moist chamber at 4°C for overnight (1 μl antibody + 200 μl PBS per slide).
6. Next day wash with PBS (3 × 5 mins).
7. Apply anti mouse and anti rabbit no. 1 Biotynalated link for 30 mins -1 hrs at RT.
   (apply about 3 drops directly on slide).
8. Wash with PBS (3 × 5 mins).
9. Apply about 3 drops directly Streptavidin HRP no. 2 for LSAB. 10-15 mins.
10. Wash with PBS (3 × 5 mins).
11. Apply DAB (5 ml DAB+5 μl H_2O_2).
12. Observe under microscope for brownish colour.
13. Dip in water for 5 mins.
14. Apply HE for 30 sec-1 min.
15. Wash several times.
16. Ion exchange water wash 1 time.
17. 80% ethanol wash for 1 min.

18. 90% ethanol wash for 1 min.
19. 100% ethanol wash for 1 min (3 times).
20. Xylene wash for 1 min (3 times), Coverslip.
21. Examine color development.

An exemplary result is shown in Figure **1**. As shown in the right portion of Figure **1**, when ascorbic acid 2-phosphate was added as an ECM synthesis promoting agent, a contractile three-dimensional tissue of the cells was only slightly observed. On the other hand, by detaching the sheet-like cells from the base of the culture dish and allowing the cells to self organize, the cells were promoted to be layered and were accelerated into a three-dimensional structure, as shown in the left portion of Figure **1**. As shown in a left portion of Figure **1**, large tissue without a hole was also produced when synovial cells were used. This tissue was thick and its extracellular matrix was rich as shown in a right portion of Figure **1**. When ascorbic acid 2-phosphate was added at a concentration of 0.1 mM or more, the formation of an extracellular matrix was promoted (Figure **2**). Figure **3** shows an enlarged view of a synthetic tissue on Day 3, 7, 14, and 21. As can be seen, after 3 days of cultre, the tissue was already so rigid that it can be detached (Figure **3**). As the number of culture days is increased, the density of the extracellular matrix fluctuates and increases.

The tissue was detached from the base of the culture dish and self-contracted. The synthetic tissue was prepared in a sheet form. When the sheet was detached from the dish and was allowed to stand, the sheet self contracted into a three-dimensional structure. It is seen that a number of layers of cells exist in the tissue.

Next, various markers including extracellular matrix markers were stained.

Figure **4** shows the result of staining extracellular matrix. It can be seen that various extracellular matrix components (collagen I, II, III, fibronectin, vitronectin, etc.) existed. Immunostaining was conducted, so that collagen I and III were strongly stained while collagen II staining was limited to a portion. By being strongly magnified, it can be confirmed that collagen was stained at a site slightly away from the nuclei, i.e., collagen was a part of the extracellular matrix. On the other hand, fibronectin and vitronectin, which are believed to be important cell adhesion molecules. By being strongly magnified, it can be confirmed that fibronectin and vitronectin were stained at a region close to nulei unlike collagen, i.e., fibronectin and vitronectin existed around the cells.

These results demonstrated that cells of at least 3 to 8 passages are preferable for production of synthetic tissue.

For comparison, a nomal tissue and a collagen sponge (CMI, Amgen, USA) were stained. Figure **5** shows the normal tissue (normal synovial membrane tissue, tendon tissue, cartilage tissue, skin, and meniscus tissue). Figure **6** shows the stained collagen sponge, which was the comparative example. From the left, fibronectin, vitronectin, negative control, and HE staining are indicated. As can be seen, the conventional synthetic tissue was not stained with fibronectin or vitronectin. Therefore, the synthetic

tissue of the present invention is different from conventional synthetic tissues. Existing collagen scaffolds do not contain fibronectin and vitronectin (adhesion agents). In view of this, the originality of the synthetic tissue of the present invention is clearly understood. No stain in found in the extracellular matrix. When the synthetic tissue of this example was compared with normal tissue, the synthetic tissue has a lower extracellular matrix density and had a structure different from normal tissue.

Further, when the synthetic tissue of the present invention was contacted with a filter paper in order to remove moisture from the tissues, the filter is adhered to the synthetic tissue, and it was difficult to manually detach the synthetic tissue of the present invention.

In order to determine the collagen concentration, the collagen content was measured. The result is shown in Figures **7** and **8**. As can be seen, the amount of hydroxyproline clearly indicates that when 0.1 mM or more ascorbic acid 2-phosphate was added, the production of collagen was significantly promoted. The amount of produced collagen is substantially proportional to the time period of culture (Figure **8**).

(Example 2: Measurement of collagen production)

Next, it was determined whether or not collagen (extracellular matrix) is sufficiently secreted after implantation of a synthetic tissue of the present invention. The following protocol was used.

<Method>

Culture periods: 3 days, 7 days, 14 days, and 21 days,

Concentrations of ascorbic acid 2-phosphate: 0 mM, 0.1 mM, 1 mM, and 5 mM

Under the above-described conditions, a synovial membrane-derived synthetic tissue was produced.

6 N HCl was added to culture medium for the synthetic tissue, followed by hydrolysis at 105°C for 18 hours. The medium was oxidized with chloramine T. Thereafter, the synthetic tissue was subjected to color development using Ehrlich's Reagent Solution (2 g of p-dimethylamino-benzaldehyde + 3 ml of 60% perchloric acid; isopropanol was diluted at 3:13), followed by measurement of absorbance.

<Results>

1) The quantities of collagen produced was dependent on the ascorbic acid concentration in the following manner: 0 mM << 5 mM < 1 mM ≤0.1 mM (Figure **7 and 8**).

2) it was demonstrated that the quantity of produced collogen is increased with an increase in the culture time period.

(Example 3: Influences of the size of a dish, the number of cells, and the number of passages)

Next, influences of the size of a dish and the number of passages were investigated.

Figure **9** shows the formation of synthetic tissues where the number of cells and the number of the passage were changed. A synthetic tissue was formed in all concentrations tested.

Under the conditions of the above-described Example 1, a similar experiment was conducted where the sizes of dishes were 35 mm, 65 mm, and 100 mm and the number of passages were 5 to 7 (Figure **10**).

The results are shown in Figures **9** and **10**. Figure **9** shows the states of synthetic tissues, where the number of passages was changed. Figure **10** shows the states of synthetic tissues, where the size of a dish was changed. As can be seen from the figures, it was demonstrated that a synthetic tissue can be formed using any size of dish and any number of passages.

As shown in Figure **9**, basically, a greater number of cells may be preferable for the purpose of matrix production. However, when an excessive number of cells were provided, the cells produced an excessive level of contraction force, so that the cell sheet was detached on the day following the start of culture. Therefore, it was demonstrated that when a larger synthetic tissue is desired, it is preferable to dessimate cells at a relatively small concentration. Particularly, in order to control the strength or the like of a synthetic tissue, a relatively small cell concentration seems to be preferable. As can be seen from the figure, when the number of passages was five, the resultant cell sheet was spontaneously detached if the cell concentration was $5.0\times10^5/cm^2$, and was not spontaneously detached if the cell concentration was $2.5\times10^5/cm^2$. Also, when the number of passages was six or more, the resultant cell sheet was spontaneously detached if the cell concentration was $7.5\times10^5/cm^2$, and was not spontaneously detached if the cell

concentration was $5.0\times10^5/cm^2$. Therefore, the production of a preferable synthetic tissue of the present invention seems to require a sufficient number of cells and a relatively great number of passages. Fourth passage cells were used to produce a trial synthetic tissue. It was spontaneously detached when the cell concentration was $40\times10^5/cm^2$. Thus, there seems to be a close relationship between the strength of a synthetic tissue and the number of passages. Various synthetic tissues can be produced, depending on the application. According to these results, cells capable of withstanding implantation seems to be obtained by culturing fifth passage cells at a concentration of $4.0\times10^5/cm^2$, however, the present invention seems not to be limited to this.

Similarly, the strength of tissues consisting of other cells is demonstrated to be able to be regulated by changing the cell concentration. Under the conditions described in Example 1, myoblasts can be used to produce a synthetic tissue and the influence of cell density on the strength of the synthetic tissue can be measured. Under the conditions described in Example 28, synovial cells can be used to produce a synthetic tissue and the influence of cell density on the strength of the synthetic tissue can be measured. Under the conditions described in Example 12, fat-derived cells can be used to produce a synthetic tissue and the influence of cell density on the strength of the synthetic tissue can be measured.

(Example 4: Measurement of mechanical properties)

In this example, cells (4×10^5 cells/cm^2) were cultured in medium containing ascorbic acid 2-phosphate for three weeks. Following detachment at 48 hours, the mechanical properties of the tissue were investigated. The protocol

will be described below.

The mechanical properties were examined by a tensile test.

Figures **11** and **12** show the outer appearance of a testing apparatus. Figure **11** shows a test piece holding portion (an original piece is shown). As shown in Figure **12**, the opposite ends of a synthetic tissue were held by the test piece holding portion. A marker was attached to the synthetic tissue for ease of measurement. Figure **13** shows the attachment of the marker. Figure **14** shows an enlarged view of the test piece holding portion. Figure **15** shows the state of the synthetic tissue after a tensile test.

A synthetic tissue was held as shown in the figures and a marker was attached to the synthetic tissue, followed by a tensile test. The maximum load was 1.89 N, and the Young's modulus was 19.2 Mega pascal. As a reference, the maximum load (tension) of cartilage is typically 0.7 and that of skin is 1.2. The Young's modulus of cartilage is 10 MPa and that of skin is 35 Mpa. Thus, it was demonstrated that the synthetic tissue of the present invention has substantially the same mechanical strength as that of skin, cartilage, or the like, and can resist surgical handling.

The results of the experiment are shown in Figures **16** and **17**. The results demonstrate that the maximum load was 1.89 N and 1.9 N, respectively. Young's modulus (tangent tensile modulus) was 19.2 MPa.

(Example 5: Determination of self-supporting ability)

Next, the self-supporting ability of a synthetic tissue of the present invention was tested. The synthetic tissue was held and tested using curved fine forceps A-11 (made of stainless steel; full length: 120 mm; curved: 20 mm, tip: 0.1 mm; manufactured by Natsume Seisakusho). It was determined by visual inspection whether or not the synthetic tissue has self-supporting ability. If the synthetic tissue was divided into a plurality of pieces, it was determined to lacking self-supporting ability. The same result was obtained when another forceps, e.g., curved fine forceps A-12-2 (made of stainless steel, full length: 100 mm; tip: 0.05 mm; manufactured by Natsume Seisakusho) were used by another experimenter performing the same expriment.

The self-supporting ability may be determined immediately after detaching a synthetic tissue off or after preserving a detached synthetic tissue.

None of the synthetic tissues comprising cardiomyocytes, myoblasts, and synovial cells, which are produced in the presence of a three-dimensional promoting agent comprising ascorbic acid as described in the above examples, had self-supporting ability. In contrast, it was already difficult to hold a synthetic tissue produced in the absence of such an agent with forceps upon detachment, so that lack of self-supporting ability was confirmed.

Therefore, 1) if a sheet is easily detached by circumferential pipetting; and 2) if the detached sheet is easily attached to a target site by lightly touching an edge thereof, the sheet spontaneously contracts to have sufficient strength.

Therefore, self-supporting ability is a property which was first obtained by the method of the present invention.

(Example 6: Osteogenic differentiation induction)

In this example, it was determined whether or not the synthetic tissue of the present invention works when osteogenesis was induced in the synthetic tissue.

It was confirmed that synovial cells can be cultured in osteogenesis induction medium (10% FBS-DMEM+0.1 μM dexamethasone, 10 mM beta glycerophosphate, 0.2 mM ascorbic acid 2-phosphate) from the beginning to produce a synthetic tissue.

Also, it was confirmed that a synthetic tissue was produced without osteogenesis induction, and thereafter, the medium was exchanged with osteogenesis induction medium and the tissue was cultured, so that calcificated bone was generated in the synthetic tissue. The result is shown in Figure **18**.

Whereas a synthetic tissue without differentiation induction appears to be transparent, an ossificated synthetic tissue has a white colour. The synthetic tissue was strongly stained with Alizarin Red, and was also strongly stained by alkali phosphatase (ALP) staining as compared to the control. Thus, it was confirmed that the synthetic tissue of synovial cells is capable of osteogenesis.

(Example 7: chondrogenesis induction)

In this example, it was determined whether or not chondrogenesis induction can be used for the production method of the synthetic tissue of the present invention.

(Culture conditions)
Cell density: 4×10^4 cells/cm^2
Conditions: CO_2 5%, air 95%, 37°C

These conditions and a chondrogenesis induction medium described below were used to produce a synthetic tissue.

Cartilage differentiation induction medium: DMEM (GIBCO), FBS (HyClone) 10%, ITS+Premix (insulin, transferrin, selenious acid) (BD Biosciences) 6.25 μg/ml, dexamethasone (Sigma) 10^{-7} M, ascorbic acid (WAKO) 50 μg/ml, pyrubic acid (SIGMA) 100 μg/ml.

The results are shown in Figure **19**. The cells were induced into cartilage. From the left, a typical medium, a chondrogenesis induction medium, a chondrogenesis induction medium+BMP-2, and a chondrogenesis induction medium+TGF-b1 were used to culture a synthetic tissue. All of the tissues were stained blue with Alcian blue staining. It was confirmed that a cartilage-like matrix production was accelerated. Such an effect is significant for cells cultured in medium containing BMP-2. The result of quantification of staining ability is shown in Figure **20**.

Expression of cartilage-associated genes (aggrecan, Col II, Sox9) in the synthetic tissue is shown in Figure **21**. When the synthetic tissue was transferred from the typical medium (leftmost column) to the chondrogenesis induction

medium (middle column), expression of the Sox9 gene, which is a chondrogenesis marker, was increased. When the synthetic tissue was further cultured in the chondrogenesis induction medium+BMP-2, expression of the collagen II gene was also increased. Thus, stronger chondrogenesis could be confirmed. Figure **22** shows the results of comparison of a chondrogenesis reaction between a monolayer culture synovial cell and a synovial cell in a three-dimensional synthetic tissue, when the same differentiation inducing stimulus was applied. When counted from the left, odd-numbered columns indicate monolayer culture, while even-numbered columns indicate three-dimensional synthetic tissue, where culture was performed under the same culture conditions. When the chondrogenesis induction medium or the chondrogenesis induction medium+BMP-2 was added as a stimulus, it was confirmed that the chondrogenesis marker gene was significantly expressed in the synthetic tissue. Thus, the three-dimensional synthetic tissue was confirmed to have strong chondrogenesis ability.

(Example 8: Repair of a pig cartilage)

Next, it was determined whether or not cartilage can be repaired. An allogenic synthetic tissue was used.

To determine the presence or absence of the adhesion capability of a synthetic tissue, an allogenic synthetic tissue was implanted onto a pig cartilage piece. The synthetic tissue was prepared under conditions where the number of cells was 4.0×10^6 cells/35-mm dish, the concentration of ascorbic acid was 1 mM, and the culture period was 7 to 14 days. A wound having a diameter of 6 mm was generated on the cartilage piece. An upper layer zone thereof was cut off from the cartilage piece using a scalpel.

Chondroitinase ABC (1 U/ml) was added. The cartilage piece was allowed to react for 5 minutes. A synthetic tissue was sized to have a diameter of 6 mm and was implanted, followed by culture for 7 days. The synthetic tissue is closely attached to the attachment surface of the cartilage piece. Fibronectin aggregated on the attachment surface (Figure **23**).

Next, pig cartilage implantation was performed. As described above, a wound having a diameter of 6 mm was created in a medial femoral condyle. An upper layer zone thereof was cut off from the cartilage piece using a scalpel. Chondroitinase ABC (1 U/ml) was added. The cartilage piece was allowed to react for 5 minutes. A allogenic synthetic tissue was sized to have a diameter of 6 mm and was implanted, followed by culture for 7 days. The results are shown in Figure **24**. Figure **25** shows a strongly enlarged view of a culture portion of a surface of the cartilage adhered to the synthetic tissue of Figure **24**. The left portion of Figure **25** is a photograph showing the result of HE staining, the middle portion is a photograph showing the result of staining with anti-fibronectin antibodies, and the right portion is a photograph showing the result of staining with anti-vitronectin antibodies. As indicated by an arrow (the interface between the synthetic tissue and the cartilage tissue), it was demonstrated that the matrix of the synthetic tissue was directly attached to the cartilage matrix, but not via cells. It is shown that fibronectin and vitronectin were accumulated at the adhesion surface. Thus, the results suggest that these adhesion molecules are involved in adhesion between a synthetic tissue and a recipient tissue. Therefore, the present invention is also characterized in that the synthetic tissue is more effectively adhered to

in vivo tissue than conventional synthetic tissues, or cells.

Further, the tissue was examined after one month of implantation. The result is shown in Figure **26**. As can be seen, it is confirmed that the synthetic tissue was biologically integrated with the cartilage injury portion and was accepted without inflammation. The surface layer portion of the synthetic tissue was made mainly of fibroblast-like cells as shown in Figure **27**. On the other hand, a deeper layer portion of the synthetic tissue was made mainly of cartiliage-like cells as shown in Figure **28**. Therefore, the implanted synthetic tissue had differentiated into cartilage-like tissue over time. No significant rejection was confirmed in any period of time, and rejection which is expected for allogenic implantation, was not observed.

Therefore, it was found that the allogenic synthetic tissue can be implanted without a side effect.

(Example 9: Repair of a pig meniscus)

Next, it was determined whether or not the synthetic tissue of the present invention is applicable to repair of meniscus.

As in the above-described Example 6, an allogenic synthetic tissue was prepared under conditions where the number of cells was 4.0×10^6 cells/35-mm dish, the concentration of ascorbic acid was 1 mM, and the culture period of time was 7 to 14 days. A portion having a diameter of 6.5 mm was removed from a meniscus (Figure **29**), and the synthetic tissue was implanted thereinto. The portion containing the implant was covered with a collagen sheet

(Nipro) for protection until the synthetic tissue was accepted (Figure **30**). The pig was kept for one month. The protocol is described below.

(Anesthesia)

A pig 15 to 17 weeks old (LWD ternary hybrid) was intramuscularly injected via the dorsal portion of its neck with 20 mg/kg Ketaral + 10 mg/kg Seractal. Thereafter, an infusion route was provided in the ear vein, and thereafter, the respiratory tract was secured using endotracheal intubation. Diprivan was continuously administered at a rate of 0.5 mg/kg/hr to maintain anesthesia. An antibiotic (Cefamezin, 1 g) was administered to prevent post-operational infection.

(Operation)

The animal was positioned and an operation portion was cleaned with a sterilized drape. A knee joint was accessed by medial para-patellar approach. After detecting the internal articular capsule, the middle portion at the medial collateral ligament (MCL) of the knee was defected. A cylinder-shaped cavity (diameter: 6.5 mm) was created using the mosaic plasty DP (Smith & Nephew) (Figure **29**). The cavity was filled with the synthetic tissue (Figure **30**), followed by the coverage with fascia. After hemostasis was confirmed, the incised internal collateral ligament was repaired, and the articular capsule, the subcutaneous tissue, and the epidermis were sutured. A cast was fixed to the knee joint in its incurvation position. The operation was ended.

(Evaluation method)

Visual inspection and histological study were performed.

(Results)

Four weeks after operation, the animals receiving the synthetic tissue was significantly repaired according to visual finding (Figure **31**) and histological finding (Figure **32**).

Remarkably, an eosin positive result was observed in the synthetic tissue four weeks after implantation. Also, the formation of a meniscus tissue-like matrix was observed and the biological integration of the synthetic tissue and its adjacent meniscus tissue was completed.

(Example 10: Repair of pig tendon/ligament tissues)

Tendon/ligament tissues were subjected to a repair operation. The state of the wound of a tendon/ligament tissue is confirmed. In this case, a portion of synovial cells are collected. The synovial cells are cultured. The cells are used to produce a synthetic tissue using a protocol as described in Example 1.

Next, by operation, the vicinity of the wound site of the tendon/ligament tissue is cut off to obtain a fresh portion, on which the above-described synthetic tissue is in turn placed. In this case, since the synthetic tissue has adhesion molecules, the synthetic tissue is adhered to the portion without suture. The protocol is described below.

(Anesthesia)

A pig 15 to 17 weeks old (LWD ternary hybrid) was intramuscularly injected via the dorsal portion of its neck with 20 mg/kg Ketaral + 10 mg/kg Seractal. Thereafter, an infusion route was provided in the ear vein, and thereafter,

the respiratory tract was secured using endotracheal intubation. Diprivan was continuously administered at a rate of 0.5 mg/kg/hr to maintain anesthesia. An antibiotic (Cefamezin, 1 g) was administered to prevent post-operational infection.

(Operation)

The animal was positioned and an operation portion was cleaned with a sterilized drape. A knee joint was accessed by medial para-patellar approach. After detecting the internal articular capsule, the middle portion of the capsule was dissected. The lower thighs were bent and laterally rotated, and were further pulled forward, so that the anterior horn portion of the internal meniscus was exposed. In this place, a cylinder-shaped cavity (diameter: 6.5 mm) was created using the mosaic plasty DP (Smith & Nephew) . The cavity was filled with the synthetic tissue . In order to protect the synthetic tissue until it was accepted, the meniscus was wrapped with a collagen sheet (Nipro) which was fixed by suture. After hemostasis was confirmed, the incised internal collateral ligament was repaired, and the articular capsule, the subcutaneous tissue, and the epidermis were sutured. A cast was fixed to the knee joint in its incurvation position. The operation was ended.

(Evaluation method)

Histological study was performed based on Frank's method (J. Orthop. Res., 13, 923-9,1995).

(Results)

According to visual finding and histological finding 6 weeks after operation, the group filled with the synthetic tissue had significantly better healing quality.

(Example 11: Repair of a pig bone)

In this example, repair of bone is experimentally conducted. Using a protocol as described in Example 1, synovial cells are collected and cultured to produce a synthetic tissue.

Next, a sheet of this synthetic tissue is applied to a bone. The synthetic tissue is applied to an affected portion mainly by covering it over a cortical bone as well as a periosteum. As a result, it is demonstrated that the synthetic tissue comprising synovial cells is effective for repair of a bone. The protocol is described below.

(Anesthesia)

A pig 15 to 17 weeks old (LWD ternary hybrid) was intramuscularly injected via the dorsal portion of its neck with 20 mg/kg Ketaral + 10 mg/kg seractal. Thereafter, an infusion route was provided in the ear vein, and thereafter, the respiratory tract was secured using endotracheal intubation. Diprivan was continuously administered at a rate of 0.5 mg/kg/hr to maintain anesthesia. An antibiotic (Cefamezin, 1 g) was administered to prevent post-operational infection.

(Operation)

The animal was positioned and an operation portion was cleaned with a sterilized drape. A second metatarsal bone was accessed from a longitudinal incised portion. The periosteum of the second metatarsal bone was ablated as much as possible so that the surface of the second metatarsal bone was exposed. A window of 1.5 cm (horizontal) × 3 cm (vertical) was created on the surface of the second metatarsal

bone using a chisel. The window was covered with the outstretched synthetic tissue. After confirming the attachment of the synthetic tissue, the the subcutaneous tissue and the epidermis were sutured. A cast is fixed to the lower thigh. The operation was ended.

(Evaluation method)
Radiography, micro CT, and histology.

(Results)
Four weeks after operation, evaluation confirmed that osteogenesis was accelerated in the window portion for the group filled with the synthetic tissue.

(Example 12: Pig fat-derived tissue)
Next, cells derived from adipose tissue were used to produce a synthetic tissue.

A) Cells were collected as follows.
1) A specimen was removed from the fat-pad of a knee joint.
2) The specimen was washed with PBS.
3) The specimen was cut into as many pieces as possible using scissors.
4) 10 ml of collagenase (0.1%) was added to the specimen, followed by shaking for one hour in a water bath at 37°C.
5) An equal amount of DMEM (supplement with 10% FBS) was added, followed by filtration using a 70 μl filter (available from Millipore or the like).
6) Cells which passed through the filter and residues which remained on the filter were placed in a 25-cm^2 flask (available from Falcon or the like) containing 5 ml of DMEM

supplemented with 10% FBS.

7) Cells attached to the bottom of the flask (including mesenchymal stem cells) were removed and subjected to the production of a synthetic tissue as follows.

B) Production of synthetic tissue

Next, the above-described fat-derived cells were used to produce a synthetic tissue. The concentrations of ascorbic acid 2-phosphate were 0 mM (absent), 0.1 mM, 0.5 mM, 1.0 mM, and 5.0 mM. The synthetic tissue was produced in accordance with the above-described method which was used to produce synovial cells (Example 1). Cells were dessimated at an intial concentration of 5×10^4 cells/cm^2. The result is shown in Figure **33**. The cells were cultured for 14 days. A synthetic tissue was also formed from an adipose tissue-derived cell and had as rich fibronectin and vitronectin as the synovial cell-derived synthetic tissue. Collagen I and III were similarly expressed richly.

C) Implantation experiment

Next, the above-described synthetic tissue is subjected to an implantation experiment in Example 8 (cartilage repair) and in Example 9 (meniscus repair). As a result, it is demonstrated that a repairing capability is possessed by the fat-derived synthetic tissue as with a synovial cell-derived synthetic tissue.

D) Differentiation induction of a fat-derived synthetic tissue into bone/cartilage

The synthetic tissue of this example was induced to differentiate into a cartilage or a bone. The results

are shown in Figure **34**. The left portion of the figure indicates the results of an osteogenesis experiment. The uppor portion indicates a synthetic tissue, while the lower portion indicates monolayer culture. The synthetic tissue had a positive reaction to Alizarin Red in an osteogenesis induction medium. Thus, osteogenesis was confirmed. The right portion indicates a chondrogenesis induction experiment. In this experiment, the synthetic tissue was differentiated with a stimulus due to chondrogenesis induction medium+BMP-2 into a cartilage-like tissue which was positive to Alcian blue. Thus, it was demonstrated that the fat-derived synthetic tissue also has the ability to differentiate into a bone and a cartilage as with a synovial cell-derived synthetic tissue.

(Example 13: Versatility of shape of synthetic tissue)

In this example, a difference in function due to the shape of a synthetic tissue is measured. The synthetic tissue may be crumpled up and implanted into an affected portion instead of using a sheet of the synthetic tissue. Thereby, it is determined whether or not a tailor-made operation can be conducted, depending on the shape or the like of a wound portion.

In this example, it is investigated whether or not a synthetic tissue can be implanted when it is in the shape of a ball, a line, or a tube. The synthetic tissue is confirmed not to require suture, since it has an adhesion molecule.

(Example 14: Treatment using a synovial cell)

In this example, a synovial cell is collected from a patient having an injured meniscus, and it is determined

whether or not the synovial cell can be used to produce a synthetic tissue.

(Collection of a human synovial cell)

A human patient, who has a clinical symptom is diagnosed by an imaging technique as having cartilage injury or meniscus injury, is subjected to arthroscopy under lumber anesthesia or general anesthesia. In this case, several milligrams of synovial membrane is collected. The collected synovial membrane is transferred to a 50-ml centrifuge tube (manufactured by Falcon) and washed with phosphate buffered saline (PBS). Thereafter, the sample is transferred to a 10-cm diameter culture dish (Falcon) and is cut into small pieces using a sterilized blade. Thereafter, 10 ml of 0.1% collagenase (Sigma) is added to the cut pieces in the dish. The dish is shaken in a constant temperature bath at 37°C for 1 hour 30 minutes. To the solution, 10 ml of medium (DMEM, Gibco) containing self-serum previously collected or bovine serum (FBS) is added to inactivate the collagenase, followed by centrifugation at 1500 rpm for 5 minutes to pellet the cells. Thereafter, 5 ml of the serum-containing medium is added again. The culture medium is passed through a 70-μl filter (Falcon). The collected cells are transferred to a 25 cm^2 flask (Falcon), followed by culture in a CO_2 incubator at 37°C.

(Subculture of a synovial cell)

During primary culture, medium is exchanged two times every week. When cells become confluent, the cells are subcultured. For initial subculture, the medium is suctioned and thereafter the cells are washed with PBS. Trypsin-EDTA (Gibco) is added to the cells which are in turn allowed to stand for 5 minutes. Thereafter, the

serum-containing medium is added and the resultant mixture was transferred to a 50-ml centrifuge tube (Falcon), followed by centrifugation at 1500 rpm for 5 minutes. Thereafter, 15 ml of the serum-containing medium is added to the pellet. The cells are placed in a 150-cm^2 culture dish (Falcon). Subsequent subculture is performed so that the cell ratio was 1:3. The same procedure is repeated up to 4 to 5 passages.

(Production of a synthetic tissue)

The synovial cell of 4 to 5 passages is treated with trypsin-EDTA. The synovial cells (4.0×10^6) are dispersed in 2 ml of medium containing 0.2 mM ascorbic acid 2-phosphate on a 35-ml culture dish (Falcon), followed by culture in a CO_2 incubator at 37°C for 7 days. As a result, a culture cell-extracellular matrix complex is formed. The complex is mechanically detached from the culture dish by pipetting the periphery thereof two or more hours before an implantation operation. After detachment, the culture cell-extracellular matrix complex contracts into a three-dimensional tissue having a diameter of about 15 mm and a thickness of about 0.1 mm.

(Example 15: Production of a synthetic tissue from a human adipocyte)

A collection-intended site (e.g., around a knee joint) from a patient under local anesthesia is resected. Several milligrams of adipocytes are collected from the site. The collected adipocytes were treated in a manner similar to that of the synovial cells. As a result, a three-dimensional synthetic tissue can be produced.

(Example 16: Implantation of a synthetic tissue into a joint cartilage injury portion)

The synthetic tissue produced in Example 14 or 15 is used for actual implantation. A human subject is subjected to lumbar anesthesia or general anesthesia. Thereafter, the inside of a joint is opened at minimum incision for arthroscopy. After detecting a cartilage injury portion, the size of the cartilage injury is measured. A circular portion of the cartilage is dissected from the bone-cartilage interface using the mosaic plasty harvesting system (Smith and Nephew) and a dental explorer, where the circular portion fully contains the injured cartilage. The synthetic tissue was implanted into the cavity in a portion of cartilage. The synthetic tissue is adhered to the base of the cavity several minutes after implantation. When an affected portion receives a high mechanical stress, the fixation of the synthetic tissue may be reinforced using fibrin glue (initial fixation is reinforced). The present invention is not limited to this. After fixation, the articular capsule, the subcutaneous tissue, and the skin are sutured collectively. After closing the incision site, the joint is fixed using a cast or an orthosis for 2 to 3 weeks. Thereafter, rehabilitation is started within a limited range of motion. When an affected portion is present in a weight-bearing joint (e.g., a knee, a ankle joint, etc.). A full load is able to be applied after 6 to 8 weeks.

As a result, symptoms are cured or ameliorated as follows: a reduction in joint pain when a load or an exercise is applied; elimination of joint effusion; recovery of a joint range of motion; recovery of muscle strength around the joint; prevention of osteoarthritis; and the like. Thus, it is observed that the synthetic tissue of the present invention has no significant side effects and improves the function of a repaired portion.

(Example 17: Implantation into a meniscus injury portion)

In this example, the synthetic tissue produced in Example 14 or 15 is actually implanted into a meniscus injury portion.

A meniscus injury portion is detected in a human subject under lumbar anesthesia or general anesthesia, using an arthroscope. A rupture portion of an injury meniscus is filled with the synthetic tissue. Thereafter, the injured meniscus and the synthetic tissue are sutured together. All surgical procedures are performed under an arthroscope. After surgery, a knee orthosis is used for 2 to 3 weeks. Thereafter, rehabilitation is started within a limited range of motion. A full weight bearing is permitted after 5 to 6 weeks.

As a result, symptoms are cured or ameliorated as follows: a reduction in joint pain when a load or an exercise is applied to the knee joint; elimination of hydrarthrosis; recovery of a joint range of motion; recovery of muscle strength around the joint; recovery of activity; doing sports again; and the like. Thus, it is observed that the synthetic tissue of the present invention has no significant side effects and improves the function of a repaired portion.

(Example 18: Implantation into an Achilles tendon)

The synthetic tissue produced in Example 14 or 15 is implanted into an Achilles tendon injury portion.

A human subject under lumbar anesthesia or general anesthesia is subjected to Achilles tendon by para-tendon

approach. The portion of degenative tear is detected and then curetted. The synthetic tissue is implanted into the portion of degenerative tear. After implantation, conventional tendon repair is performed. In addition, the surface layer of the repaired portion is covered with the synthetic tissue, which is in turn sutured and fixed thereto. After closing the incision site, a cast is fixed to the lower limb for 4 weeks. A full weight bearing is permitted after 6 to 8 weeks.

As a result, symptoms are cured or ameliorated as follows: recovery of activity level (from walking to a sport level); a reduction in pain; and a decrease in possibility of re-rupture. Thus, it is observed that the synthetic tissue of the present invention has no significant side effects and improves the function of a repaired portion.

(Example 19: Treatment of intractable pseudarthrosis)

In this example, intractable pseudarthrosis is treated using the synthetic tissue produced in Example 14 or 15. A feature of intractable pseudarthrosis is that a periosteum, which is a source of supplying cells in a bone fracture therapy, is severely damaged and lost. Implantation of the synthetic tissue is considered to be approparate in such a case.

A bone fracture portion is opened in a human subject under anesthesia. Thereafter, the bone fracture portion is curetted. After the remaining portion is fixed with a plate or an intramedullary nail, the injured periosteum is covered with the synthetic tissue. The synthetic tissue is sutured and fixed to adjacent periosteum tissue. After closing the

incision site, the joint adjacent to the bone fracture portion is fixed with a cast for 3 to 4 weeks. In the case of a lower limb bone, full weight bearing is permitted after 6 to 8 weeks.

As a result, symptoms are cured or ameliorated as follows: elimination of pain; recovery of muscle strength around the joint; and recovery of an activity level. Thus, it is observed that the synthetic tissue of the present invention has no significant side effects and improves the function of a repaired portion.

(Example 20: Implantation into a rotator cuff injury portion)

In this example, a synthetic tissue is implanted into a rotator cuff injury portion. The synthetic tissue is produced as described in Example 1. Under general anesthesia, the rotator cuff injury portion is detected by transdeltoid approach.

After detecting the rotator cuff injury portion, the portion is curetted and is subjected to a typical rotator cuff repair operation. Thereafter, the surface layer of the repaired rotator cuff portion is covered with the synthetic tissue. After closing the incision site, the shoulder joint is fixed with an orthosis for 2 to 3 weeks. Thereafter, rehabilitation is started within a limited range of motion. After 6 weeks, full range of motion is permitted.

As a result, symptoms are cured or ameliorated as follows: remission of shoulder pain (particularly, night pain); recovery of a joint range of motion; recovery of muscle strength around the shoulder; and recovery of activity. Thus,

it is observed that the synthetic tissue of the present invention has no significant side effects and improves the function of a repaired portion.

(Example 21: Study on the possibility of cell differentiation induction before and after production of a synthetic tissue)

In this example, a synthetic tissue is produced using a human synovial cell.

The production process of the synthetic tissue using a human synovial cell is shown in the upper portions of Figures **35** and **36**. Figure **35** shows production of a synthetic tissue after a human synovial cell is subjected to differentiation induction. Figure **36** shows that a synthetic tissue is produced before the tissue is subjected to differentiation induction. The differentiation induction is performed by culturing a human synovial cell in DMEM medium containing 0.1 μM dexamethasone, 10 mM β-glycerophosphate, and 50 μg/ml ascorbic acid 2-phosphate for 14 days. The synthetic tissue is stained with Alzarin red and alkali phosphatase (ALP). The results of the staining are shown in the lower portions of Figures **35** and **36**. As can be seen from Figure **35**, in either case, the synthetic tissue is produced and exhibits an osteogenic reaction positive to the Alzarin red and ALP staining. Therefore, it is demonstrated that the differentiation induction of a tissue can be performed either before or after production of a synthetic tissue.

(Example 22: Study on timing of differentiation for production of a synthetic tissue in the case of human cells)

In this example, a synthetic tissue was produced using

cells derived from adipose tissue.

A) The cells were collected as follows.

1) A specimen was collected from a fat-pad of a knee joint.

2) The specimen was washed with PBS.

3) The specimen was cut into as many pieces as possible.

4) 10 ml of collagenase (0.1%) was added, followed by shaking in 37°C water bath for one hour.

5) An equal amount of DMEM (supplemented with 10% FBS) was added. The resultant mixture was passed through a 70-μl filter (available from Millipore, etc.).

6) Cells passing through the filter and cells remaining on the filter were cultured in 25-cm^2 flask containing 5 ml of DMEM medium supplemented with 10% FBS.

7) The cells (including a mesenchymal stem cell) attached to the base of the flask were used to produce a synthetic tissue as follows.

B) Production of a synthetic tissue

Next, the fat-derived cells were used to produce a synthetic tissue. Ascorbic acid 2-phosphate was used at a concentration of 0 mM (absence), 0.1 mM, 0.5 mM, 1.0 mM, or 5.0 mM. The production was conducted in accordance with the method for producing a synthetic tissue from a synovial cells (Example 1). The cells were disseminated at an initial density of 5×10^4 cells/cm^2.

The cells were used to study the importance of the differentiation timing using the conditions as described in Example 21.

As a result, it was similarly demonstrated that the differentiation timing has no particular influence on the adipocyte-derived synthetic tissue of the present invention.

(Example 23: Confirmation of biological integration)

It is known that conventional collagen gel does not always achieve biological integration after implantation. In this example, a conventional collagen gel (3% type I collagen, Koken, Tokyo, Japan) was used. Synovial cells ($1x10^5$ cells/ml) were embedded in the gel. The resultant gel was implanted into a cavity in a portion of cartilage. As a result, as can be seen from Figure **37**, the integration between the collagen gel and its adjacent cartilage was insufficient, so that a crack was observed (arrow in Fig 37).

On the other hand, when a synthetic tissue of the present invention as produced in Example 1 is introduced into a pig, biological integration is histologically established as shown in Figure **38**.

(Example 24: Study on conditions for detachment during production of a synthetic tissue)

In this example, it was determined whether or not chemical detachment can be used instead of physical detachment (mechanical detachment (e.g., pipetting, etc.)) during the production of the synthetic tissue of the present invention.

(Conditions for culture)

Cell density: $4x10^4$ cells/cm^2

Conditinos: CO_2 5%, air 95%, 37°C

Medium: DMEM/F12 (FBS 10%) supplemented with 10 ng/ml TGFβ1.

This medium was used to conduct culture under the conditions described in Examples 14 and 15 to produce a synthetic tissue.

When TGF-β was added, the monolayer culture cells could be more easily detached from the culture dish.

Medium: DMEM (GIBCO), FBS (HyClone) 10%, ITS+Premix (insulin, transferrin, selenious acid) (BD Biosciences) 6.25 μg/ml, dexamethasone (Sigma) 10^{-7} M, ascorbic acid (WAKO) 50 μg/ml, pyrubic acid (SIGMA) 100 μg/ml.

The results are shown in Figures **19** and **39**. The rightmost column in Figure **19** shows the case where TGF-β was added. In this case, cells were detached from a culture dish during monolayer culture. Therefore, a synthetic tissue could not be satisfactorily produced. Figure **39** shows the result of a tissue which was detached without a physical stimulus when TGF-β was added in monolayer culture. These results indicate that TGF-β has the effect of detaching culture cells.

(Example 25: Actin regulatory agent)

Dihydrocytochalasin B and Y27632 (Yamanouchi Pharmaceutical), which are known to have an actin depolymerizing function, were used to study their influence on the contraction of a synthetic tissue.

A synovium-derived synthetic tissue was produced by monolayer culture. The tissue was detached from a culture

dish. The tissue was cultured in medium in the presence of dihydrocytochalasin B (3μM) and Y27632 (10μM). The transition of the radius of the tissue is shown every unit culture time in Figure **40**. As can be seen from the figure, contraction was inhibited by the addition of these actin depolymerizaing agents. Dihydrocytochalasin B and Y27632 are representative exemplary actin polymerization inhibitors. It will be understood by those skilled in the art that other actin polymerization inhibitors, such as cytochalasin D and the like, have a similar function.

(Example 26: Production of an artificial bone/cartilage column as a complex of a synthetic tissue and an artificial bone)

A 12-well culture dish was used to produce a synthetic tissue.

A column-like artificial bone (NEO BONE: MMT) having a diameter of 5 mm × 6 mm was placed in a 96-well culture dish. The synthetic tissue was implanted onto the artificial bone. 100 μl of medium (DMEM, 10% FBS) was placed in each well of the dish, followed by culture for 2 hours. As a result, the synthetic tissue was attached to the artificial bone, thereby obtaining a tissue complex.

This complex was cultured in cartilage induction medium (DMEM, 10% FBS, ITS+Premix, sodium pyrubate, ascorbic acid 2-phosphate, 500 ng/ml BMP-2) for 14 days.

The result is shown in Figure **41**.

As can be seen from Figure **41**, it is demonstrated that the synthetic tissue of the present invention was

satisfactorily adhered to the other synthetic tissue (i.e., the artificial bone). Therefore, it will be understood that the synthetic tissue of the present invention can be combined with other synthetic tissues into a tissue complex.

(Example 27: Composite tissue obtained by attaching a synthetic tissue to a collagen scaffold)

In this example, a microfibrous collagen medical device (specifically, a collagen synthetic tissue (CMI (Collagen Meniscal Implant) collagen sponge, Amgen,USA)) was attached to a synthetic tissue instead of NEO BONE in Example 26. The result is shown in Figure **42** (enlarged photograph). The synthetic tissue of the present invention is observed to be biologically integrated with the surface of the CMI. Thus, it was demonstrated that a microfibrous collagen medical device, which is a conventional synthetic tissue, can be combined with the synthetic tissue of the present invention to obtain a tissue complex.

(Example 28: Production of a synthetic tissue using a myoblast)

In this example, an influence of ascorbic acid or a derivative thereof on the production of a synthetic tissue when a myoblast was used, was studied. The synthetic tissue was produced as in Example 1.

After the myoblast was well grown, 5×10^6 myoblast cells were cultured to form a synthetic tissue. For culture, SkBM Basal Medium (Clonetics (Cambrex)) was used. Next, ascorbic acid 2-phosphate (0.5 mM), a magnesium salt of ascorbic acid 1-phosphate (0.1 mM), and L-ascorbic acid Na (0.1 mM) were added to the medium. After four days of culture, the tissue was detached. As a control, a synthetic tissue

was produced in medium without ascorbic acids.

(Results)

When ascorbic acids were used, the synthetic tissue was easily detached as compared to when the ascorbic acid-free culture system was used. Also, in the ascorbic acid-free culture system, the tissue was cultured to about several millimeters. When the tissue exceeded such a level, a crack or the like occurred in the tissue so that the tissue did not grow satisfactorily. In addition, it was substantially difficult to detach the tissue. Thus, no implantable synthetic tissue was produced (Figure **43**). In contrast, the synthetic tissue of the present invention, which was cultured in medium containing ascorbic acids, was grown to a size which allows implantation, and was easily isolated (Figure **44**). Biological integration was investigated, so that extracellular matrices were highly interacted (Figure **45**).

(Example 29: Effect of a synthetic tissue in the presence of ascorbic acids)

The synthetic tissue of Example 28, which was produced in the presence of ascorbic acids, was implanted into a dilated cardiomyopathy rat. In 28 rats, the left anterior descending (LAD) was ligated for two weeks to produce injured hearts. The synthetic tissue of the present invention was implanted into some of the injured hearts, while the synthetic tissue of the present invention was not implanted into the other injured hearts. As controls, rats without injury to their hearts were obtained.

The rats were anesthetized and operated. The heart function of the rats was monitored on Day 14 and 28 after

surgery. A ultrasonic instrument (Sonos 5500) having an anular array converter operating at 12 MHz was used to perform endocardiography. Parasternal minor axis imaging and parasternal major axis imaging were performed in a B-imaging mode and an M-imaging mode. In addition to the anterior wall pressure, general parameters (e.g., left ventricular telediastolic diameter, left ventricular telesystolic diameter, internal diameter contraction rate, and ejection fraction) were measured.

Two and four weeks after implantation, the rats were sacrificed with an excessive amount of pentobarbital. The heart was dissected, fixed with 10% formalin, and embedded in paraffin. In a low temperature bath, the heart was cut along the longitudinal axis thereof from the base to the apex to prepare a series of sections having a thickness of 5 mm. Thereafter, the sections were treated for standard histology.

All of the rats with implants were completely cured, and survived for substantially the same period of time as normal rats. Therefore, it was demonstrated that the present invention can completely cure diseases, which are conventionally said to be intractable, in the presence of a specific ECM synthesis promoting agent.

(Example 30: Combined therapy)

A combined therapy of the synthetic tissue produced in the examples and a gene therapy was performed. The combined therapy was intended to promote vascularization in a portion which a synthetic tissue was implanted; promotion of acceptance of an implanted synthetic tissue; and suppression of cell necrosis in a synthetic tissue.

(Methods)

A hemagglutinating virus of Japan (HVJ)-liposome complex was prepared in accordance with Kaneda Y., Iwai K., Uchida T., Increased expression of DNA co-introduced with nuclear protein in adult rat liver. Science, 1989;243:375-378. The procedure will be briefly described below. A DNA solution (200 µl) was added, followed by shaking for 30 seconds. The solution was allowed to stand at 37°C in a constant temperature bath for 30 seconds. This step was performed 8 times. Thereafter, ultrasonication was performed for 5 seconds, followed by shaking for 30 seconds. BSS (0.3 ml) was added, followed by shaking at 37°C in a constant temperature bath. Inactivated HVJ was added. The mixture was placed on ice for 10 minutes. The mixture was then shaken at 37°C in a constant temperature bath for one hour. A 60% sucrose solution (1 ml) and a 30% sucrose solution (6 ml) were layered in a centrifuge tube. A HVJ liposome solution was placed on top of the layered sucrose solution. Additional BSS was added to the tube. Centrifugation was performed at 62,800 g at 4°C for 1.5 hours. A solution immediately above the 30% sucrose solution layer was recovered. The solution was preserved at 4°C and was used for gene introduction.

About 0.2 ml of Sendai virus liposome-plasmid complex (including 15 µg of human HGF cDNA) was injected into a cardiac infarction region. For a control group, an empty vector was introduced into a heart muscle having infarction. The human HGF concentration of heart tissue was measured with an enzyme linked immunosolvent assay (ELISA) using an anti-human HGF monoclonal antibody (Institite of Immunology, Tokyo, Japan) (Ueda H., Sawa Y., Matsumoto K. et al., Gene Transfection of Hepatocyte Growth Factor

Attenuates reperfusion Injury in the Heart, Ann. Thorac. Surg., 1999, 67:1726-1731). The synthetic tissue produced in Example 30 was used. The cardiac infarction models produced by ligating LAD were subjected to three different therapies: 1) a cell sheet group; 2) a gene therapy group; 3) a combined therapy group; and 4) a control group. Changes in heart function and cardiomuscular tissue were studied.

(Results)

For the synthetic tissue implanted group and the combined therapy group, the contractibility and expansibility of the heart were ameliorated. In addition, for the combined therapy group, it can be confirmed that vasculization was observed in the cardiac infarction portion, and the acceptance of implanted cells was improved.

(Conclusion)

By combining a synthetic tissue and a gene therapy, the decreased heart function ameliorating effect, the vasculization effect, and the cell protecting effect are obtained, so that a higher level of amelioration of the decreased heart function can be observed.

Although certain preferable embodiments have been described herein, it is not intended that such embodiments be construed as limitations on the scope of the invention except as set forth in the appended claims. Various other modifications and equivalents will be apparent to and can be readily made by those skilled in the art, after reading the description herein, without departing from the scope and spirit of this invention. All patents, published patent applications and publications cited herein are incorporated by reference as if set forth fully herein.

INDUSTRIAL APPLICABILITY

The present invention usefully provides a basic therapeutic method, technique, pharmaceutical agent, and medical device for diseases which are conventionally difficult to treat. Particularly, the present invention provides an epoch-making therapy and prevention because it promotes recovery to a substantially native state. The present invention also provides a pharmaceutical agent, cell, tissue, composition, system, kit, and the like, which are used for such an epoch-making therapy and prevention.

There is a demand for repair and regeneration of joint tissues, mainly including bones and cartilages which are targeted by the present invention. The number of bone fracture patients, which are targeted by bone regeneration, accounts for several hundreds of thousands per year. It is also said that there are 30 million potential patients having osteoarthritis which is targeted by the cartilage regenerative therapy. Thus, the potential market is huge. The present invention is also highly useful for peripheral industries. Acute competition has been started in the regenerative medical research on joint tissues, mainly including bone and cartilage. The synthetic tissue of the present invention is a safe and original material made of cells collected from an organism, such as a patient or the like, and is highly useful in view of the lack of side effects or the like.